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October 2015


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# EMERGING INFECTIOUS DISEASES

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## On the Cover

**John James Audubon (1785–1851). *Cow-pen Bird*, Plate XCIX (99)** Engraved from watercolor. 19.5 × 12.25 in. / 49.53 × 31.12 cm. Image from Archives Service Center, University of Pittsburgh, Pittsburgh, PA, USA.

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## Synopses



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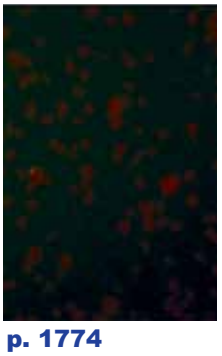

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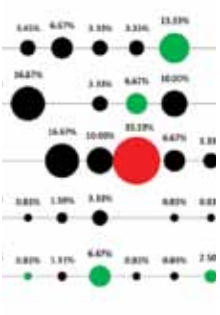

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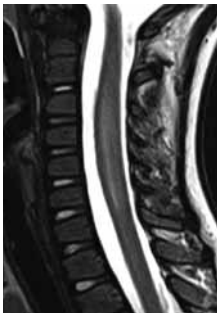

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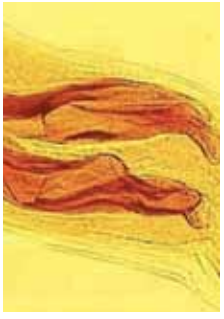

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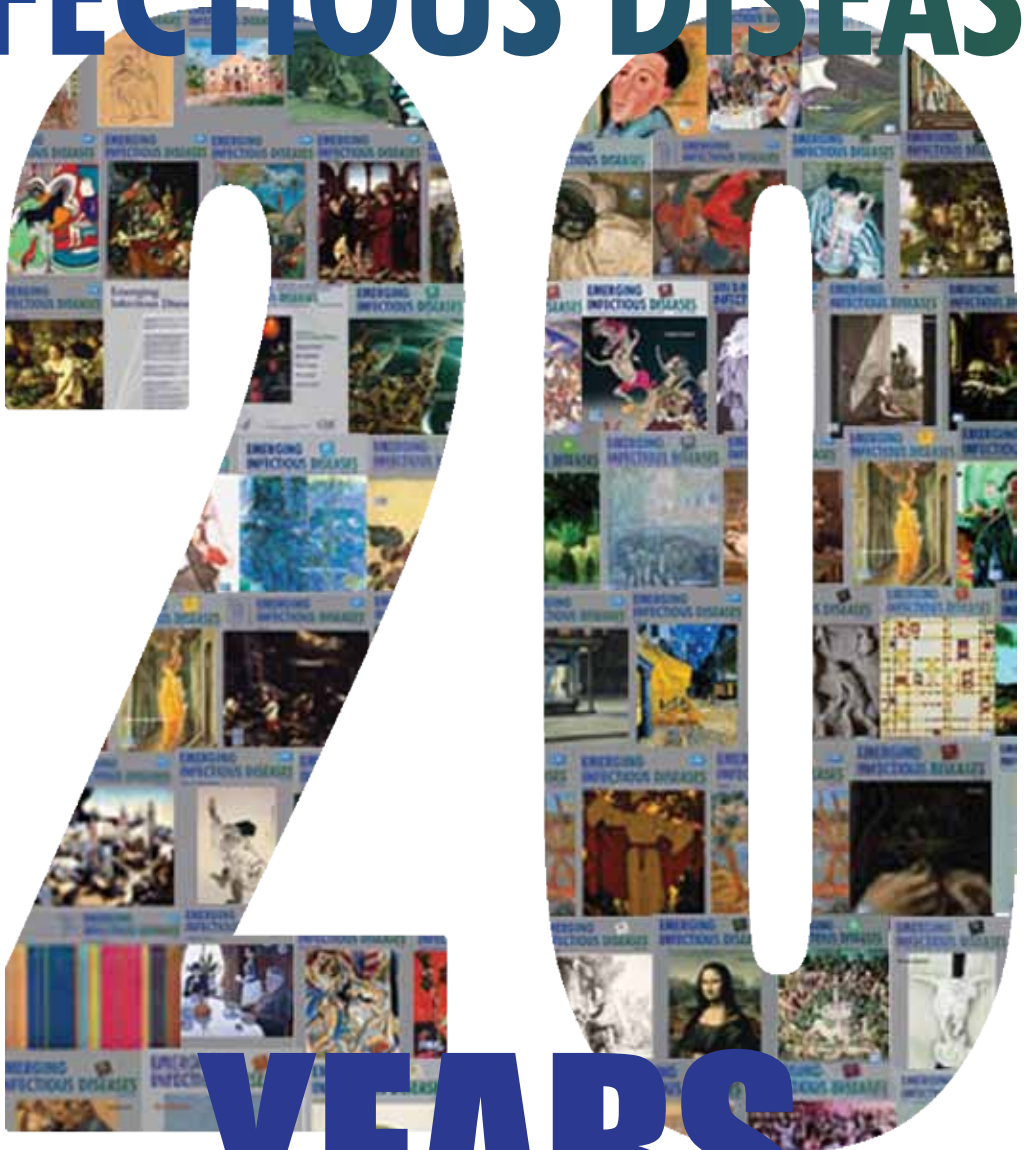

# 20 YEARS

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# Pacific Broad Tapeworm *Adenocephalus pacificus* as a Causative Agent of Globally Reemerging Diphyllobothriosis

**Roman Kuchta, Marcus Enrique Serrano-Martínez, Tomas Scholz**

The Pacific broad tapeworm *Adenocephalus pacificus* (syn. *Diphyllobothrium pacificum*) is the causative agent of the third most common fish-borne cestodosis among humans. Although most of the nearly 1,000 cases among humans have been reported in South America (Peru, Chile, and Ecuador), cases recently imported to Europe demonstrate the potential for spread of this tapeworm throughout the world as a result of global trade of fresh or chilled marine fish and travel or migration of humans. We provide a comprehensive survey of human cases of infection with this zoonotic parasite, summarize the history of this re-emerging disease, and identify marine fish species that may serve as a source of human infection when eaten raw or undercooked.

Infection with the tapeworm *Adenocephalus pacificus* (syn. *Diphyllobothrium pacificum*) (Cestoda: Diphyllobothriidea) was described by Nybelin in 1931 in the Juan Fernández fur seal, *Arctocephalus philippii*, from waters of the Juan Fernández Islands off the coast of Chile. This parasite has been reported among 9 of 16 species of extant otariid seals and has wide distribution, mostly in the Southern Hemisphere (*1*). The convoluted taxonomic history of the genus, which was synonymized with *Diphyllobothrium* Cobbold, 1858, has been recently reviewed by Hernández-Orts et al. (*1*), who resurrected the name *Adenocephalus* Nybelin 1931, on the basis of molecular and morphological evidence and transferred *D. pacificum* back to *A. pacificus*. However, in this article, we use the established term "diphyllobothriosis" to describe infection with parasites in this genus and for human disease caused by *A. pacificus*.

In addition to otariids, infections with *A. pacificus* have been reported among humans and dogs who consumed raw or undercooked marine fishes (*2*). The first 2 human cases of diphyllobothriosis caused by this species were briefly reported from Callao, Lima, Peru, in 1957 (*3*). Another case in a student from Trujillo, Peru was erroneously reported as having been caused by *Diphyllobothrium latum* (Linnaeus, 1758) (*4*). However, the eggs of this tapeworm were found in coprolites and mummified humans in several archeological sites in Peru and northern Chile (*5*).

So far, ≈50 records of diphyllobothriosis caused by *A. pacificus* have been published; many of them were published in regional journals that are difficult to obtain (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/10/15-0516-Techapp1.pdf). Neither a synthesis of these cases nor an exhaustive list of fish that are potential intermediate hosts has been published. We conducted an extensive search of the literature and examined extensive samples of *A. pacificus* (*1*,*6*) to present a comprehensive synopsis of the human disease caused by *A. pacificus*, including data on the geographic distribution of human cases and a survey of potential fish hosts of this zoonotic parasite that serve as a source of human infection.

## History of *Adenocephalus pacificus* Diphyllobothriosis among Humans

### Archeological Data

The Pacific broad tapeworm *A. pacificus* seems to have coexisted with humans at least since the early Neolithic period, as evidenced by the recovery of diphyllobothriidean eggs in archeological samples such as coprolites or mummies (*5*). The first findings of cestode eggs in coprolites from South America were identified as those of *Diphyllobothrium* sp., *D. latum*, or *D. trinitatis* Cameron, 1936 (*5*). However, this species identification is questionable, because evidence shows that *D. latum* originally occurred in the Northern Hemisphere only (*2*); *D. trinitatis* is a *species inquirenda* (i.e., a species of uncertain taxonomic status because of insufficient available data).

The first archeological records of *A. pacificus* found in coprolites were from the coastal site of Los Gavilanes in Peru, dated from 2850 to 2700 BCE (*7*), and from the site of Tiliviche in northern Chile (Iquique), with Chinchorro culture dating from 4110 to 1950 BCE (*8*) (Figure 1). The latter site lies 40 km from the Pacific coast at an

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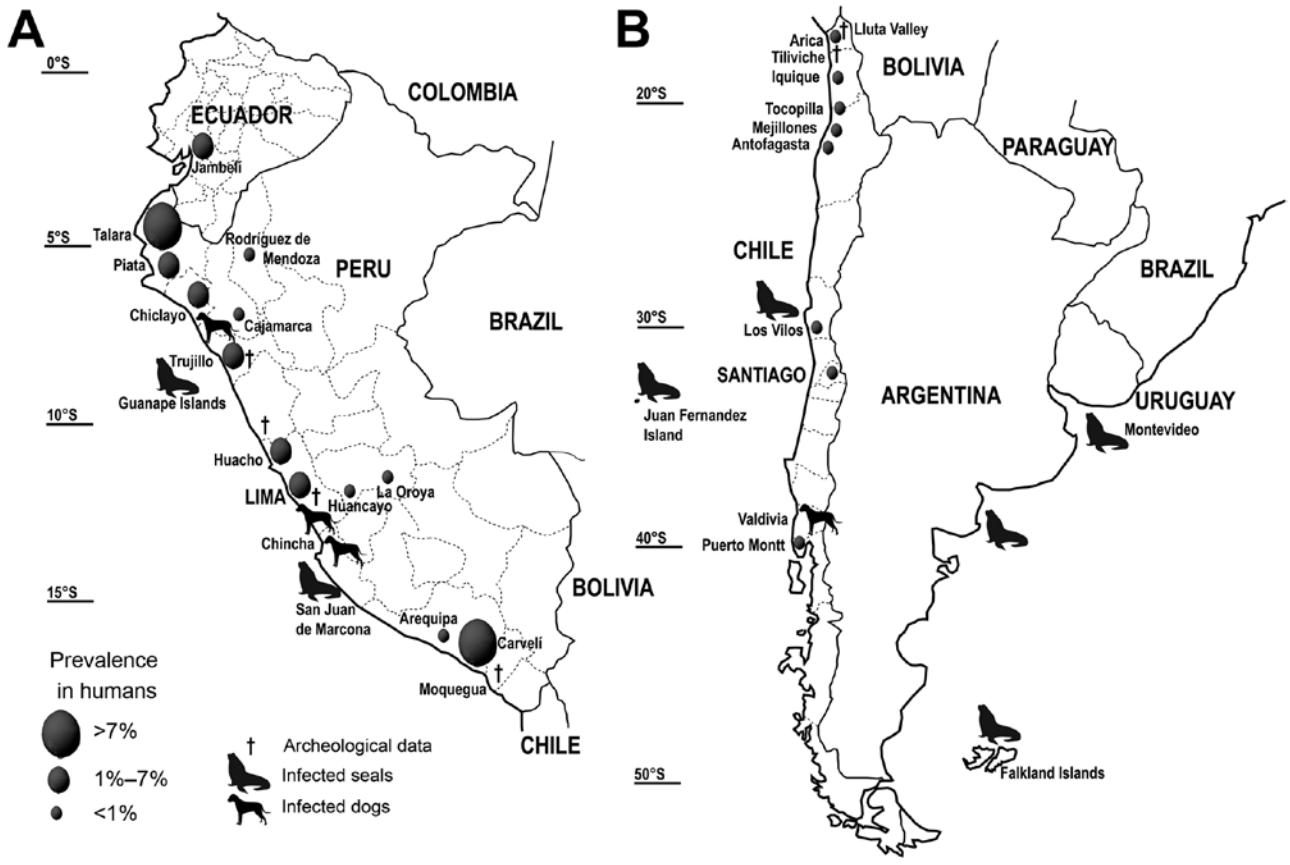


**Figure 1.** Distribution of the Pacific broad tapeworm *Adenocephalus pacificus* among humans and wild animals on the A) northern and B) southern Pacific coast of South America.

altitude of 950 meters, which may demonstrate that diphyllobothriosis was not limited to the coastal areas, either as a result of import of marine fish or movement of infected persons from the coast (*8*,*9*). The presence of *A. pacificus* eggs were then confirmed in other mummified bodies of humans in Chinchorro, dated 3050–2050 BCE (*10*). (Figure 1).

The oldest record of *A. pacificus* from the South American continent was dated to the Neolithic period, as long ago as 8000 BCE, from an unknown locality on the northern Peruvian coast (*11*), but this dating later appeared to be incorrect because radiocarbon dates were not precise (K. Reinhard, pers. comm.). The eggs of the Pacific broad tapeworm were reported from Preceramic cultures in Peru (2850–2500 BCE) in Huarmey Valley and Huaca Prieta (*7*), as well as from Ceramic cultures (*5*). Several records are also known from the pre-Inca (Chiribaya culture, 800–1400 BCE) and Inca (1476–1534 BCE) eras in Peru and northern Chile (*5*) (Figure 1). A comparison of data from the pre-Inca and Inca eras in San Geronimo, Chile, indicated that the number of cases among humans increased when Incas conquested this region (*12*).

The archeological record of eggs of *A. pacificus* in the skeleton of a child found on Adak Island in Alaska, reported by Bouchet et al. (*13*), is unlikely accurate because the shape of the egg does not correspond to that typical of *A. pacificus*. Some *Diphyllobothrium* species may have been misidentified (*2*).

### Modern Times and the Present

No reliable reports of diphyllobothriosis are available for most of the modern or post-colonial period, after 1500 CE (*5*). The first confirmed case of human infection with *A. pacificus* in modern times was identified in 1967 by Baer (*14*), who disputed a previously published report of a tapeworm misidentified as *D. latum* by Miranda et al. (*4*) and reported an additional 7 cases (online Technical Appendix Table 1). Also in 1967, Rêgo (*15*) published a case report of a student from Peru who was in Argentina and was infected with a tapeworm identified as *Lueheella* sp. (syn. of *Spirometra*). Re-examination of voucher specimens from the Helminthological Collection of the Instituto Oswaldo Cruz in Rio de Janeiro, Brazil (CHIOC nos. 30161 and 30162) revealed that the tapeworm was

misidentified and was actually *A. pacificus* (R. Kuchta and T. Scholz, unpub. data).

Since the 1950s, ≈1,000 cases of human infection with *A. pacificus* tapeworms have been reported. These reports are chronologically summarized in the online Technical Appendix Table 1, together with all published records of *A. pacificus* adults from all species of definitive hosts.

The number of reported cases during 1957–2015 in individual decades fluctuated irregularly, partly depending on whether comprehensive research reports of numerous human cases or only individual case reports were published in a given decade (online Technical Appendix Table 1). The number of human infections increased most considerably during 1981–1990. In the 21st century, the number of reported cases declined conspicuously, but this may be related to an inexplicable gap in reporting diphyllobothriosis in Latin America after 1990 rather than to an actual decline of human infections. Diphyllobothriosis is not considered to represent a serious health problem in Peru, especially when its effect on human health is compared to that caused by cysticercosis, which is widely distributed in that country.

## Distribution of Cases among Humans

*A. pacificus* tapeworms are the most widely distributed endoparasitic helminth of seals, and infections occur in temperate areas of the North and South Pacific regions and in some southern temperate zones of the Atlantic and Indian oceans (*1*) (online Technical Appendix Table 1). In contrast, human infections have been reported almost exclusively from the Pacific coast of South America, mainly from Peru and, in a relatively few records, from Chile and Ecuador (Figure 1).

### South America

*A. pacificus* is the most common cestode species that causes fish-borne diseases in South America (*2*). Other diphyllobothriid species, such as *D. latum*, and sporadically, *D. dendriticum* (Nitzsch, 1824), have been rarely reported as adults in human infections or as plerocercoids (larvae) from fishes in Chile, Argentina, and Brazil. Identification was verified by molecular data for only 3 cases of *D. latum* infection (*16*).

### Peru

On the coast of Peru, ≈1,000 cases of infection with *A. pacificus* tapeworms have been reported since 1957 (*17*) (online Technical Appendix Table 1). Prevalence has been as high as 7.5% in some regions but is ≈2% in most regions (*18*,*19*) (Figure 1). Some studies showed prevalence of up to 83% (*20*), but these data were calculated on the basis of small sample sizes.

Most cases are associated with the coast, but some have been reported from inland provinces such as Amazonas and Junín (Figure 1; online Technical Appendix Table 1). One case was reported from a town in the Andean region at an altitude of 3,460 m (*21*).

### Other Countries in South America

Only 18 cases of human infection with *A. pacificus* tapeworms have been reported from Chile since 1975 (*16*,*22*) (online Technical Appendix Table 1), most from Antofagasta in northern Chile. One case of uncertain origin was reported from Los Lagos in Puerto Montt, located in southern Chile (*23*) (Figure 1). The cases from Chile were proposed to be related to the *El Niño* Southern Oscillations (ENSO) phenomenon, presumably caused by changes in water temperatures that result in the southern displacement of marine fish native to Peruvian waters and the creation of conditions favorable for the overgrowth of copepods (*22*,*24*). However, no evidence supports this theory, and reports of human cases do not correspond to the years of the ENSO phenomenon (online Technical Appendix Table 1).

A few cases were also reported from Ecuador, where the northernmost case among humans (latitude 3°S) was diagnosed (Figure 1). However, only 1 epidemiologic study reporting diphyllobothriosis is available (*25*); of 373 fecal samples examined, 13 (3.5%) were infected.

### Outside South America

Few records exist of *A. pacificus* infections in humans outside South America. Cases among 6 humans were reported from Japan (*26*; online Technical Appendix Table 1), but these cases have not been confirmed by molecular data. In Japan, as many as 11 species of diphyllobothriid cestodes have been reported to infect humans (*2*), and misidentification with other species cannot be ruled out. The first case was described by Kamo et al., who examined tapeworms found by Sunagawa in a man, 35 years of age, from Okinawa Prefecture (*27*). Another case may have been imported: the infected person, a seaman from Kitakyushu City, served as a crew member on trips along the coast of Africa (online Technical Appendix Table 1). The most recent human case reported in Japan was diagnosed in a man from Matsuyama City, Ehime Prefecture (*26*). *A. pacificus* was also reported in fur seals in Japan (*28*), but all reported human cases are limited to southern Japan (Okinawa, Kyushu, Shikoku), which is outside of the area of distribution of fur seals. The origin of human infections in Japan is thus unclear.

The distribution area of *A. pacificus* tapeworms among otariids is much wider than that in humans, which apparently represent incidental, atypical definitive hosts. The tapeworm is distributed globally, documented by confirmed records from the North Pacific (Canada, Far East Asia, United States), South Pacific (Peru, Chile, New Zealand), South Atlantic (Argentina, South Africa, Uruguay), and South Indian (Australia) Oceans (*1*) (online Technical Appendix Table 1).

However, very few autochthonous human cases have been reported from the northern hemisphere.

In 1937, Rutkevich described 2 new species of *Diphyllobothrium*: *D. giljacicum* and *D. luxi*, from the Nivkh people on the Sakhalin Island (Far East Russia), collected during expedition in 1928 (*29*). *D. luxi* is most probably the synonym for *D. nihonkaiense*; however, *D. giljacicum* described from 10 Nivkh (also known as Gilyak) people on the west coast of the Sakhalin Island seems to be closely related to *A. pacificus*. The longest specimen was 3.63 meters, and the worm showed several similarities with *A. pacificus*: shape of bothria and scolex, wide and short neck, separated opening of cirrus-sac and vagina, and small eggs (≤57 μm; eggs of *D. nihonkaiense* and *D. latum* are usually >60 μm). Both species described by Rutkevich (*29*) were incorrectly synonymized as *D. latum* (*30*). With exception of the sporadic cases from the Sakhalin Island (*29*), there are no records of *A. pacificus* infection in humans in North America and Far East Asia**,** even though fur seals are heavily infected with this cestode on the northern Pacific coasts of these continents (*1*) and other diphyllobothriid cestodes such as *D. nihonkaiense* occur in man relatively frequently (*2*).

## Recent Cases among Humans

The ability of the *A. pacificus* tapeworm to expand its distribution area globally is demonstrated by infections of humans in Spain, which have recently been confirmed by molecular data (*6*,*31*). The source of human infection in Europe remains to be clarified, but commercial import of marine fish stored on ice from areas to which the parasite is endemic, such as Chile or Ecuador, may be a plausible explanation. Spain is the third largest importer of fish and seafood in the world and imports fresh or chilled fish (i.e., those that may harbor infective plerocercoids of diphyllobothriid tapeworms) (*2*). The import of fish products from South America is critical in the spread of the parasite; countries to which *A. pacificus* tapeworm is endemic (i.e., Ecuador, Chile, and Peru) represent major exporters (*6*). Travel-associated cases or migration of humans may also result in distribution of diphyllobothriid cestodes to area outside endemic zones.

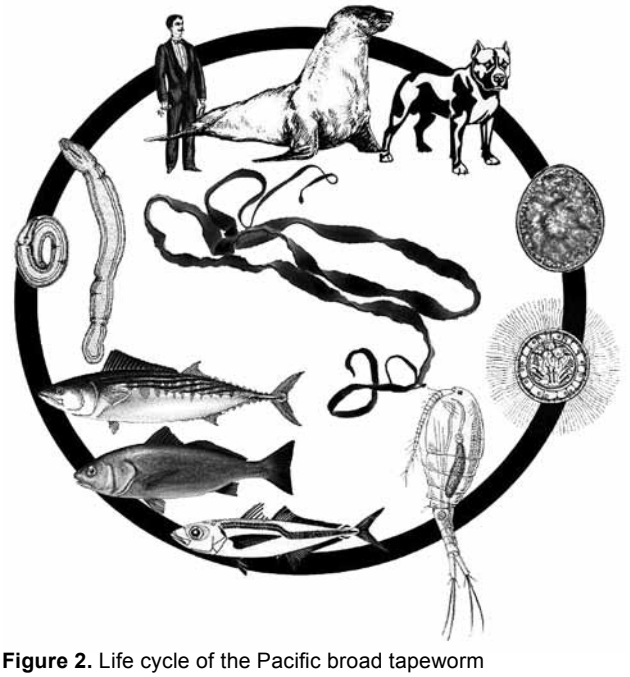

**Figure 2.** Life cycle of the Pacific broad tapeworm *Adenocephalus pacificus*. From top: definitive hosts (otariid seals, humans, dogs); egg; coracidium; potentional first intermediate host (copepod); second intermediate hosts (*Sarda chiliensis*, *Sciaena deliciosa*, *Trachurus murphyi*); encysted plerocercoids in body cavity of fish.

## Source of Human Infection

The life cycle of *A. pacificus* tapeworms is not completely known, and no data on the first intermediate hosts are available. Because marine mammals serve as definitive hosts, the life cycle is undoubtedly completed in the sea, unlike the freshwater cycle of most other human-infecting diphyllobothriids (*2*). Thus, we may assume that the cycle includes marine copepods as the first intermediate hosts, marine fish as the second intermediate hosts, and fish-eating mammals, including humans, as the definitive hosts (*2*) (Figure 2).

Humans become infected with *A. pacificus* tapeworms when they eat raw or insufficiently cooked marine fish or food items made from these fish. In coastal regions of Peru, dishes made with raw fish, such as cebiche, tiradito, and chinguirito, are popular and represent the main source of human infections (*2,32*). Several marine fish inhabiting waters off the Peruvian coast have been reported as potential intermediate hosts of *A. pacificus*, but their actual spectrum has never been critically reviewed.

The plerocercoids of *A. pacificus* are encysted in membraneous cysts in the viscera, on the peritoneum or in the stomach wall; some have also been found outside of the intestinal wall and in the gonads (*33*) (Figure 3). However, they have never been found in musculature. The cysts are thin-walled, oval, pearly white, and measure 2–4 mm in diameter (*33*,*34*). Excysted plerocercoids are relatively large (total length of 4–22 mm), and their anterior end (future scolex) possesses distinct bothria measuring 0.5–1.4 mm in length (Figure 3); the surface of plerocercoids is wrinkled and covered with microtriches ≈4 μm long. The species identification of these plerocercoids as *A. pacificus* was confirmed by sequencing of the *cox1* gene (*1*).

Baer (*33*) first reported plerocercoids of *A. pacificus* from 2 species of marine fish caught on the coast of Peru: the Eastern Pacific bonito *Sarda chiliensis* and Atlantic Spanish mackerel *Scomberomorus maculatus*. However,

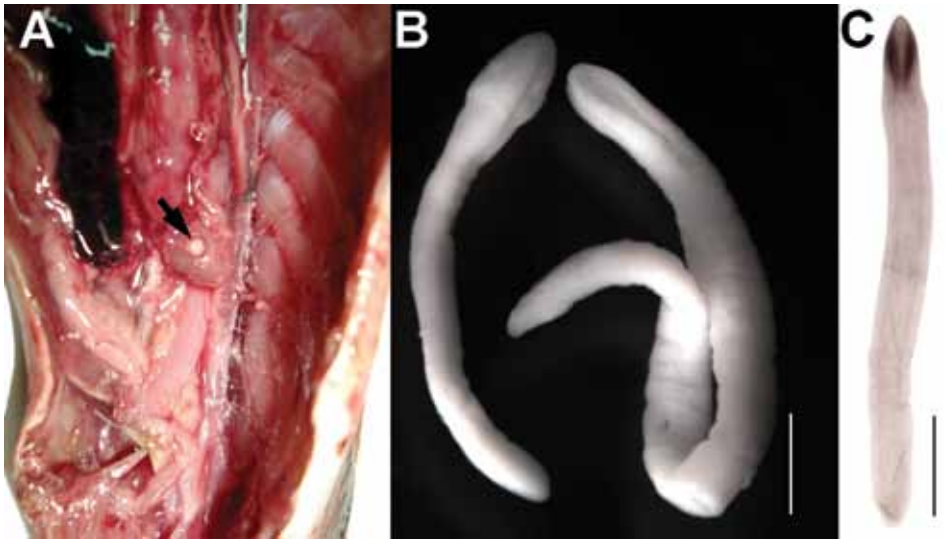


**Figure 3.** Photomicrograph of plerocercoids of *Adenocephalus pacificus* from *Sarda cholensis* off Peru. A) Body cavity with encysted plerocercoid (arrow). B) Liberated plerocercoids under stereomicroscope. Scale bar indicates 1 mm. C) Whole mount of the plerocercoid. Scale bar indicates 2 mm.

the first morphological description of plerocercoids supposedly belonging to *A. pacificus* was made by Tantalean (*33*), who found plerocercoids in the peritoneum and gonads of the lorna drum, *Sciaena deliciosa,* and the Peruvian banded croaker, *Paralonchurus peruanus*. Plerocercoids of other diphyllobothriid cestodes may also use marine fish as intermediate hosts (*33*). To date, plerocercoids allegedly from *A. pacificus* tapeworms were found in 21 fish species of 12 phylogenetically unrelated and ecologically distant families of different orders, including 1 shark species (online Technical Appendix Table 2). However, only 8 fish species were confirmed as suitable second intermediate hosts of *A. pacificus* by experimental infections of dogs or genotyping (online Technical Appendix Table 2). Other fish species may serve as second intermediate hosts, as can be assumed from anamnestic data of humans infected with *A. pacificus* tapeworms (online Technical Appendix Tables 2, 3), but their actual role in transmission must be confirmed by finding *A. pacificus* plerocercoids. Documented prevalence of fish infection with *A. pacificus* plerocercoids has seldom exceeded 20% (online Technical Appendix Table 2). We dissected 79 fish of 5 species collected off the coast of Lima, Peru and found 66 plerocercoids in the body cavity of 2 species with intensity of 2–3 per fish (online Technical Appendix Table 2, Figure 3).

## Pathology and Clinical Signs

Diphyllobothriosis is notoriously known as a potential cause of vitamin B12 avitaminosis and megaloblastic anemia (*35*). However, this effect of the parasite on its human host is rare, and most cases in which these conditions were reported as human infections with *D. latum* tapeworms occurred in Finland after World War II (*2*). Clinical symptoms of diphyllobothriosis are usually mild; the most common clinical signs are abdominal discomfort or pain and diarrhea (*2*).

Clinical signs related to human *A. pacificus* infection are poorly known and have been studied in more detail only 3 times, all in Peru: Lumbreras et al. (*36*) studied 32 cases, Medina Flores et al. (*17*) 21 cases, and Jiménez et al. (*37*) 20 patients. Additionally, 37 individual symptom reports have also been analyzed (online Technical Appendix Table 3). From a total of 110 case-patients, 18 had no clinical signs, but most of the symptoms were mild or nonspecific, such as abdominal pain (n = 74), diarrhea (n = 37), weight loss (n = 17), nausea (n = 11), or vomiting (n = 5) (online Technical Appendix Table 3). Megaloblastic anemia and vitamin B12 deficit were reported in 1 and 5 patients, respectively (*36–38*).

Typically, *A. pacificus* infections are registered after spontaneous elimination of tapeworms from the patient (online Technical Appendix Table 3). Diphyllobothriosis caused by *A. pacificus* infection has sporadically reported in AIDS patients; García et al. (*39*) found only 4 (2%) of 217 AIDS patients infected with this tapeworm, but diarrhea may be a consequential complication and causes malabsorption and malnutrition among these patients.

## Diagnosis and Control

Differential diagnosis of diphyllobothriid cestodes from human-infecting species of *Taenia* is easy and straightforward because they differ by the position of gonopores (median in diphyllobothriids versus lateral in taeniids). In contrast, identification of most diphyllobothriid cestodes from clinical material is usually impossible based only on their morphologic characteristics (*2*). The *A. pacificus* tapeworm represents one of the few exceptions because its proglottids possess papilla-like protuberances separated by semicircular pits between the genital atrium and the anterior margins of segments (*1*); these protuberances are absent in other species that cause diphyllobothriosis. In addition,

*A. pacificus* eggs are somewhat smaller and more spherical than those of human-infecting species of *Diphyllobothrium*, and the worm's genital atrium has an almost equatorial position, which distinguishes it from *Diphyllobothrium*, in which it has a more anterior position (*1*,*6*).

The only way to exactly determine the species of the causative agent and thus the origin of the infection is through sequencing and analysis of the parasites' genes. To facilitate differential identification of morphologically indistinguishable human-infecting broad fish tapeworms (*D. latum*, *D. dendriticum*, *D. nihonkaiense*) and *A. pacificus* in clinical samples, a diagnostic method has been developed and optimized by Wicht et al. (*40*). The method is based on results of a multiplex PCR amplification of a selected gene (*cox1*) and does not involve sequencing; thus, this method represents a substantively less costly and easily interpretable approach to be used routinely, mainly by medical diagnostic laboratories.

Treatment of patients who have diphyllobothriosis is simple and highly effective by a single dose of niclosamide (2 g in adults) or praziquantel (*2*,*36*). Lumbreras et al. (*36*) sufficiently treated 32 case-patients by using a single dose of 10 mg/kg of praziquantel. However, a single administration of a 25–50 mg/kg dose is usually applied to ensure complete expulsion of diphyllobothriid tapeworms (*2*).

The imports of fishery products are subject to official certification. The national authorities must also guarantee that the relevant hygiene and public health requirements are met. The provisions are aimed at ensuring high standards and at preventing any contamination of the product during processing. Scholz et al. and Kuchta et al. compiled information for processing fish to avoid survival of plerocercoids of diphyllobothriid cestodes (*2*).

## Conclusions

Human disease caused by infection with the Pacific broad tapeworm *A. pacificus* is endemic to the Pacific coast of South America, and most (>99%) clinical cases are reported from Peru. However, this tapeworm species occurs globally, and recent cases of human infection in Europe illustrate that more attention should be paid to this emergent fishborne zoonosis (*6*). The increasing popularity of eating raw or undercooked fish, import of fresh chilled or insufficiently frozen fish, and traveling and migration of humans represent risk factors that may contribute to a more global expansion of fishborne parasitoses caused by diphyllobothriid cestodes, including *A. pacificus*.

Samples of tapeworms found in humans should be processed adequately to enable molecular diagnosis and thus identification of the sources of human infection and the geographic origin of parasite infective stages (plerocercoids). Therefore, positive fecal samples or pieces of the strobila should be placed immediately to 96%–99% molecular-grade ethanol (i.e., not technical, denaturated ethanol). Samples should never be fixed with formalin unless part of the same sample is also fixed with ethanol. Fixed samples should be sent to a specialized parasitological laboratory, in which molecular and morphological identification can be performed. The laboratory of the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, České Budějovice, Czech Republic, is able to analyze and reliably identify clinical samples of diphyllobothriid cestodes free of charge. We highly recommend that representative samples be deposited in a parasite collection so that specialists can conduct further study if necessary.

For a better control of zoonotic disease caused by the Pacific broad tapeworm, gaps in our knowledge of its biology, epidemiology, and transmission should be filled. In particular, a limited knowledge of the fish intermediate hosts impedes a more effective control of fishery products and thus restriction of export of those fish that may harbor *A. pacificus* plerocercoids. Additionally, little is known about the factors that have contributed to the almost complete absence of human diphyllobothriosis outside South America, especially in the North Pacific, where *A. pacificus* tapeworms occurs frequently in fur seals but no human cases have been confirmed. The use of molecular markers for reliable identification of clinical samples should become an obligatory practice because it is necessary for a better understanding of the epidemiology of this zoonotic parasite.

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Dr. Kuchta is researcher at the Institute of Parasitology of the Biology Centre, Czech Academy of Sciences. His research interests include parasite biology, phylogeny, and molecular diagnosis of parasitic helminths, mainly diphyllobothriosis and sparganosis.

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# Characteristics and Factors Associated with Death among Patients Hospitalized for Severe Fever with Thrombocytopenia Syndrome, South Korea, 2013

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In South Korea, nationwide surveillance for severe fever with thrombocytopenia syndrome (SFTS) began during 2013. Among 301 surveillance cases, 35 hospitalized case-patients in 25 areas were confirmed by using virologic testing, and 16 (46%) case-patients subsequently died. The SFTS cases occurred during May–November and peaked during June (9 cases, 26%). The incidence of SFTS was higher in the southern regions of South Korea. Age and neurologic symptoms, including decreased level of consciousness and slurred speech, were heavily associated with death; neurologic symptoms during the first week after disease onset were also associated with death. Although melena was common among patients who died, no other hemorrhagic manifestations were substantively more common among those who died. No effective treatments, including ribavirin, were identified. Expansion of SFTS surveillance to include the outpatient sector and development of an antibody test would enhance completeness of SFTS detection in South Korea.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease that is caused by a novel phlebovirus in the *Bunyaviridae* family. This virus has been named severe fever with thrombocytopenia syndrome virus (SFTSV). The disease was first reported during 2009 in China, where it is most prevalent in the Hunan, Hubei, and Shandong provinces, which are located at a similar latitude to that of South Korea and Japan (*1*). SFTS is mainly transmitted to humans by SFTSV-infected ticks, most frequently *Haemaphysalis longicornis* (*2*). However, person-to-person transmission by direct contact with infected blood or body fluid has also been reported (*3*,*4*).

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The clinical symptoms of SFTS include fever; gastrointestinal (GI) symptoms (e.g., diarrhea and vomiting); leukocytopenia and thrombocytopenia; bleeding tendency; and neurologic symptoms. The incubation period for SFTS is 1–2 weeks, and illness progresses through 3 stages: fever, multiorgan dysfunction, and convalescence. No effective treatment for SFTS has been established (*5*).

In South Korea, the laboratory diagnostic system for SFTS was established during March 2013, and the presence of SFTSV among *H. longicornis* ticks was confirmed by using samples from ticks that were collected during 2011–2012 (*6*). Based on the nationwide surveillance reports for SFTS and its designation as a notifiable infectious disease, the first SFTS case was retrospectively confirmed in May 2013, after the patient's death in 2012 (*7*). However, because of the novel nature of this system, the clinical and demographic characteristics of patients in South Korea infected with SFTS are not well understood. Therefore, this study evaluated the characteristics and factors that were associated with SFTS-related fatalities in South Korea, as reported during 2013.

## Methods

### Surveillance System and Case Definition

A passive hospital-based surveillance system for SFTS was initiated nationwide in South Korea during March 2013. Physicians were advised to request SFTSV testing for patients with known clinical manifestations of SFTS, including fever (body temperature ≥38.0°C), thrombocytopenia, leukocytopenia, and GI symptoms. Each patient's enrollment for SFTSV testing was dependent on the physician's clinical suspicion of SFTS. Serum samples from 301 patients admitted on the basis of physician referrals at 125 hospitals throughout Korea were collected and tested. Case-patients were defined as patients who had clinical symptoms and were confirmed to have SFTSV by virologic testing at the Korea Centers for Disease Control and Prevention

(KCDC). This study did not require an institutional ethics review because it was conducted under the Infectious Disease Control and Prevention Act in South Korea.

### Laboratory Testing

All acute-phase serum samples were tested to detect the SFTSV medium segment gene by one-step reverse transcription PCR, by using DiaStar 2× OneStep reverse transcription PCR Pre-Mix Kit (SolGent, Daejeon, South Korea), as described (*8*). The PCR primers were MF3 (5′-GATGAGATGGTCCATGCTGATTCT-3′) and MR2 (5′-CTCATGGGGTGGAATGTCCTCAC-3′). The PCR conditions were an initial step of 30 min at 50°C for reverse transcription; 15 min at 95°C for denaturation; 35 cycles of 20 s at 95°C, 40 s at 58°C, and 30 s at 72°C; and a final extension step of 5 min at 72°C.

### Epidemiologic Investigation

We also performed an epidemiologic investigation of patients in whom SFTS was suspected immediately after the SFTSV testing was requested by their physicians. KCDC Epidemic Intelligence Service officers interviewed the patients and their physicians using a standardized questionnaire that evaluated demographic characteristics, exposure history, clinical symptoms, and laboratory results. Patients were also questioned regarding their exposure history within the month before their onset of symptoms. Multiple activities were documented, including agricultural and forestry work, mountain climbing, and visits to a family grave. The date of the tick bite was self-reported when the patient was aware of a bite or if the patient participated in only 1 exposure-related activity over a short period of time. In addition, after the death or discharge of confirmed case-patients during September 2013–January 2014, the clinical course and prognosis were investigated by reviewing medical records. The date for clinical suspicion of SFTS was recorded as the date reported to the KCDC.

### Statistical Analysis

We obtained median regional population data during 2013 from Statistics Korea (http://www.kostat.go.kr) to calculate the regional incidences during 2013. These incidences were then overlaid on a map of South Korea by using biogeographic information system software (DIVA-GIS 7.5; http://www.diva-gis.org). We used the Fisher exact test or the Mann-Whitney U test to compare the prognoses of patients with SFTS. All statistical analyses were performed by using SAS software version 9.2 (SAS Institute, Cary, NC, USA), and statistical significance was set at $p<0.05$.

### Results

During 2013, a total of 36 hospitalized patients were confirmed to have SFTS. One case was excluded because the patient's disease onset occurred during 2012. Thus, 35 cases from 20 hospitals were included in our analysis.

### Demographic Characteristics

Symptom onset occurred during May–November 2013; a peak of symptoms among 9 (26%) patients occurred in June. Similar patterns were observed among the SFTS case-patients in our study (Figure 1, panel A) and the number of *H. longicornis* ticks that were collected each month by Park et al. during 2011–2012 (*6*) (Figure 1, panel B). Peaks of symptoms among patients occurred in June 2013, and peaks of the number of ticks collected occurred in May 2011 and May 2012.

The overall incidence of SFTS during 2013 was 0.7 cases/1 million persons. Geographic differences were documented in the incidences in 25 cities; higher incidences

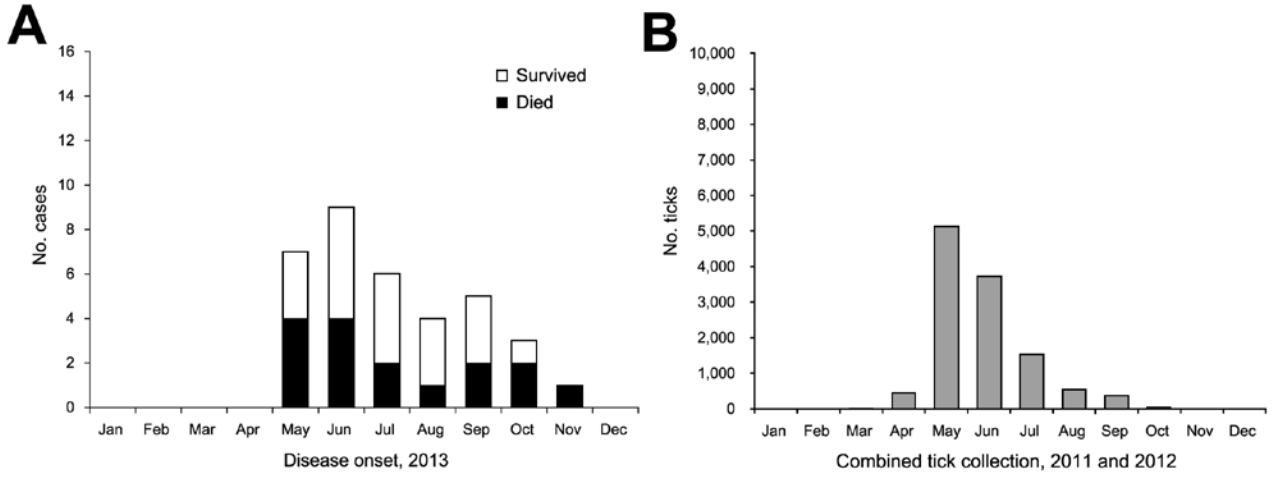


**Figure 1.** Comparison of epidemic curve for severe fever with thrombocytopenia syndrome cases identified during 2013 and the number of *Haemaphysalis longicornis* ticks collected per month during 2011 and 2012, South Korea. A) Number of cases of severe fever with thrombocytopenia syndrome, by month of onset. B) Combined number of *H. longicornis* ticks collected, by month (*6*).

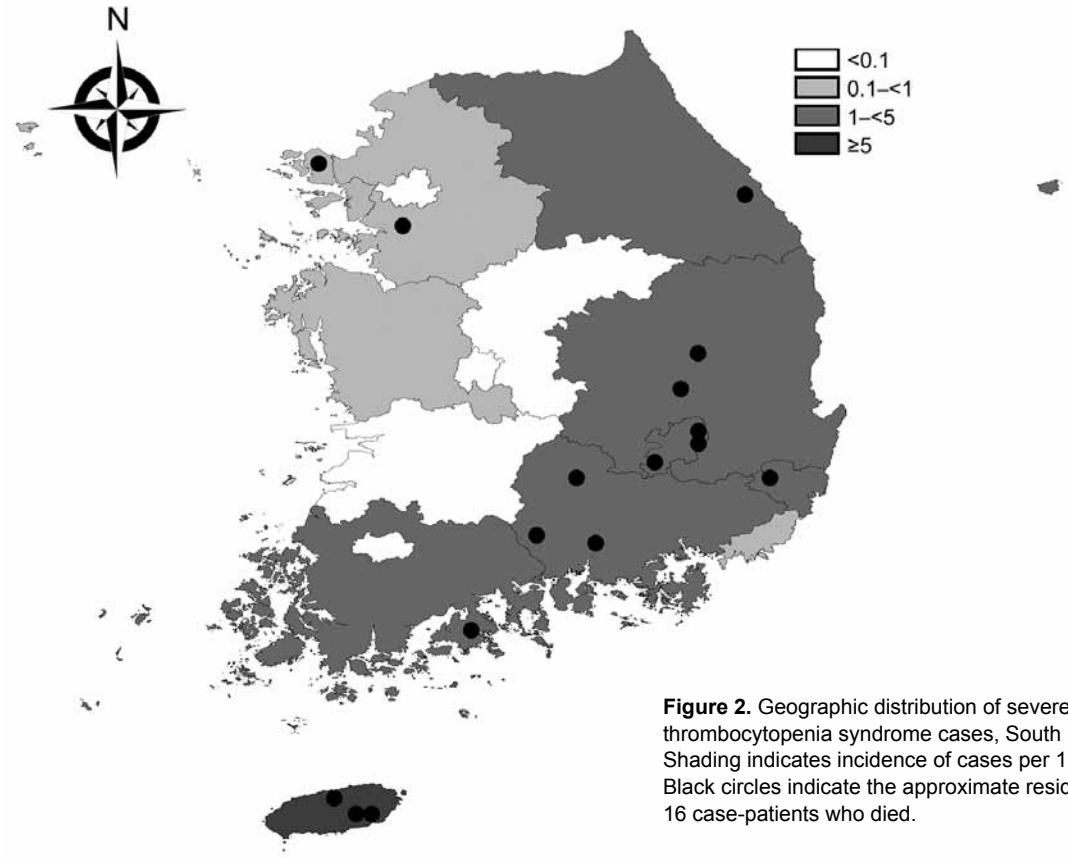


**Figure 2.** Geographic distribution of severe fever with thrombocytopenia syndrome cases, South Korea, 2013. Shading indicates incidence of cases per 1 million residents. Black circles indicate the approximate residential regions of the 16 case-patients who died.

were observed in the southern regions, including Jeju Province (Figure 2).

The case-patient group comprised 18 women (51%) and 17 men (49%). The median age was 69 (range 28–84) years, and age was significantly higher among those who died (range 62–82 years; p = 0.026). Twenty-six (74%) case-patients resided in rural areas, and 25 (71%) were farmers. A trace of a tick bite was found on 11 (31%) case-patients, and 4 (13%) had recognized the tick bite before their hospital admission. The most common exposure-related activity was agricultural work (n = 20, 61%), followed by forestry work (n = 7, 21%), mountain climbing (n = 6, 18%), and visiting a family member's grave (n = 3, 9%). The agricultural work was performed in dry fields by 16 (80%) of 20 case-patients and forestry work was done in orchards by 5 (71%) of 7 case-patients (Table 1).

The incubation period was available for 8 patients; the median was 9 (range 5–16) days. Median time from symptom onset to hospital admission was 4 (range 1–9) days and from symptom onset to clinical suspicion was 7 (range 3–22) days. We found no substantive differences in time from symptom onset to admission and to clinical suspicion between the group of persons who died and those who recovered. Twenty-four (69%) of the 35 patients with SFTS were admitted to intensive care units, and 16 (46%) died. The median survival time among the 16 was 10.5 (range 4–32) days; 11 (69%) patients died within 2 weeks after symptom onset. Among patients who survived, median time from symptom onset to discharge was 18 (range 10–49) days (Table 1).

The cohort included 1 family cluster, which consisted of an uncle and nephew. They did not live in the same village and did not have contact with each other after symptom onset. However, within 2 weeks before symptom onset, both had visited the same family member's grave on a mountain and had mowed the grass.

### Clinical Characteristics and Prognoses

The clinical characteristics of the 35 SFTS case-patients during the course of the illness are shown in Table 2. All patients experienced fever or chills, and other common symptoms included GI symptoms (n = 33, 94%), neurologic symptoms (n = 27, 77%), fatigue (n = 26, 74%), myalgia (n = 19, 54%), and hemorrhagic manifestations (n = 12, 34%). Among the 32 patients with medical records for the first 7 days after disease onset, all patients had a fever within 7 days of symptom onset. A high fever (≥39°C) was documented for 7 (22%) patients, although no significant difference was observed

**Table 1.** Characteristics of hospitalized case-patients with confirmed severe fever with thrombocytopenia syndrome, by outcome, South Korea, 2013*

| Characteristics | No. (% or range) | | | p value |
|---|---|---|---|---|
| | Total, n = 35 | Died, n = 16 | Recovered, n = 19 | |
| Sex | | | | |
| M | 17 (49) | 7 (44) | 10 (53) | 0.738 |
| F | 18 (51) | 9 (56) | 9 (47) | |
| Age, y | 69 (28–84) | 73.5 (62–82) | 61 (28–84) | 0.026 |
| Residence | | | | |
| Rural | 26 (74) | 11 (69) | 15 (79) | 0.700 |
| Urban | 9 (26) | 5 (31) | 4 (21) | |
| Occupation | | | | |
| Farmers | 25 (71) | 12 (75) | 13 (68) | 0.723 |
| Others | 10 (29) | 4 (25) | 6 (32) | |
| Medical history | | | | |
| Diabetes mellitus | 11 (31) | 5 (31) | 6 (32) | 1.000 |
| Hypertension | 18 (51) | 9 (60) | 9 (47) | 0.738 |
| Hepatitis B | 2 (6) | 2 (13) | 0 (0) | 0.202 |
| Tuberculosis | 1 (3) | 1 (6) | 0 (0) | 0.457 |
| Trace of tick bite | 11 (31) | 5 (31) | 6 (32) | 1.000 |
| Recognition of tick bite, n = 32 | 4 (13) | 2 (14) | 2 (11) | 1.000 |
| Exposure-related activities, n = 33† | | | | |
| Agricultural work | 20 (61) | 9 (60) | 11 (61) | 1.000 |
| Forestry work | 7 (21) | 4 (27) | 3 (17) | 0.674 |
| Mountain climbing | 6 (18) | 1 (7) | 5 (28) | 0.186 |
| Visits to family members' grave | 3 (9) | 2 (13) | 1 (6) | 0.579 |
| Time elapsed, d | | | | |
| From onset to admission | 4 (1–9) | 3.5 (1–7) | 4 (2–9) | 0.659‡ |
| From onset to clinical suspicion§ | 7 (3–22) | 6.5 (3–11) | 7 (3–22) | 0.612‡ |
| From onset to death | ND¶ | 10.5 (4–32) | ND | ND |
| From onset to discharge | ND | ND | 18 (10–49) | ND |

*ND, no data available.
†Fisher exact test was used to compare groups, unless otherwise indicated.
‡Multiple responses were allowed.
§Mann-Whitney U test was used to compare groups.
¶Days to clinical suspicion were determined based on the day of report to the Korea Centers for Disease Control and Prevention.

related to occurrence of fever among those who died and those who recovered (p = 0.195). Lymphadenopathy was identified by physical examination on admission on the neck, face, left thigh, left axilla, or left inguinal area of 5 (14%) patients.

The most common GI symptoms among all patients were diarrhea (n = 22, 63%), anorexia (n = 21, 60%), nausea (n = 16, 46%), and vomiting (n = 15, 43%). Both diarrhea and vomiting were reported for 26 (74%) patients; most (25/26, 96%) experienced diarrhea or vomiting within 1 week after symptom onset, and 18 (51%) patients had diarrhea or vomiting at the time of admission. However, no substantive differences in the occurrence of GI symptoms were observed for those who died compared with those who recovered.

Decreased level of consciousness (n = 26, 74%) was the most frequent neurologic symptom, followed by slurred speech (n = 9, 26%) and convulsion (n = 8, 23%). Neurologic symptoms occurred at a median of 6 days after symptom onset (range 2–10 days). On admission, 3 patients (9%) had neurologic symptoms. The group of case-patients who died had a significantly higher number of members who had neurologic symptoms (p = 0.047) and exhibited significantly more frequent neurologic symptoms that occurred within 7 days (p = 0.002). Decreased level of consciousness (p = 0.050) and slurred speech (p = 0.022) were significantly more common among those who died, although the frequency of convulsion was similar for both groups (p = 0.105).

The only hemorrhagic manifestation that occurred with a significant difference among those who died and those who recovered was melena (p = 0.035). None of the treatments of SFTS appeared to be effective, including the use of ribavirin, intravenous immunoglobulin, plasmapheresis, or continuous renal replacement therapy. Fungal pneumonia (n = 2, 6%) and cerebral hemorrhage (n = 2, 6%) were observed as complications among those who died.

### Laboratory Features and Prognoses

The laboratory data from the 32 patients with available medical records for the first 7 days after symptom onset are shown in Table 3. All patients had thrombocytopenia (platelets $<150 \times 10^9$/L) and leukocytopenia (leukocytes $<4 \times 10^9$ cells/L) on admission. The median minimum platelet count was marginally lower among those who died ($34 \times 10^9$/L), compared with that among those who recovered ($47.5 \times 10^9$/L; p = 0.054). The maximum serum

**Table 2.** Clinical characteristics of hospitalized case-patients with confirmed severe fever with thrombocytopenia syndrome, by outcome, South Korea, 2013*

| Characteristics | No. (%) case-patients | | | p value† |
|---|---|---|---|---|
| | Total, n = 35 | Died, n = 16 | Recovered, n = 19 | |
| Fever/chills | 35 (100) | 16 (100) | 19 (100) | 1.000 |
| Fever, n = 32 | | | | |
| ≤39.0°C | 25 (78) | 9 (64) | 16 (89) | 0.195 |
| >39.0°C | 7 (22) | 5 (36) | 2 (11) | NA |
| Fatigue | 26 (74) | 11 (69) | 15 (79) | 0.700 |
| Myalgia | 19 (54) | 9 (56) | 10 (53) | 1.000 |
| Headache | 12 (34) | 4 (25) | 8 (42) | 0.476 |
| Cough | 7 (20) | 3 (19) | 4 (21) | 1.000 |
| Sputum | 9 (26) | 3 (19) | 6 (32) | 0.460 |
| Lymphadenopathy on physical examination | 5 (14) | 1 (6) | 4 (21) | 0.347 |
| Lymph node enlargement found by CT, n = 32 | 20 (63) | 7 (54) | 13 (68) | 0.473 |
| Gastrointestinal symptoms | | | | |
| Overall | 33 (94) | 15 (94) | 18 (95) | 1.000 |
| Anorexia | 21 (60) | 8 (50) | 13 (68) | 0.317 |
| Nausea | 16 (46) | 5 (31) | 11 (58) | 0.176 |
| Abdominal pain | 9 (26) | 6 (38) | 3 (16) | 0.245 |
| Diarrhea | 22 (63) | 12 (75) | 10 (53) | 0.293 |
| Vomiting | 15 (43) | 6 (38) | 9 (47) | 0.734 |
| Diarrhea/vomiting | 26 (74) | 13 (81) | 13 (68) | 0.460 |
| Neurologic symptoms | | | | |
| Overall | 27 (77) | 15 (94) | 12 (63) | 0.047 |
| Within 7 d after disease onset | 20 (57) | 14 (88) | 6 (32) | 0.002 |
| Slurred speech | 9 (26) | 7 (44) | 2 (11) | 0.050 |
| Decreased level of consciousness | 26 (74) | 15 (94) | 11 (58) | 0.022 |
| Convulsions | 8 (23) | 6 (38) | 2 (11) | 0.105 |
| Hemorrhagic manifestations | | | | |
| Overall | 12 (34) | 8 (50) | 4 (21) | 0.090 |
| Gross hematuria | 2 (6) | 1 (6) | 1 (5) | 1.000 |
| Petechiae | 3 (9) | 1 (6) | 2 (11) | 1.000 |
| Gingival bleeding | 5 (14) | 3 (19) | 2 (11) | 0.642 |
| Hematemesis | 2 (6) | 1 (6) | 1 (5) | 1.000 |
| Hematochezia | 1 (3) | 1 (6) | 0 (0) | 0.457 |
| Melena | 4 (11) | 4 (25) | 0 (0) | 0.035 |
| Treatment | | | | |
| Ribavirin | 9 (26) | 6 (38) | 3 (16) | 0.245 |
| IVIG | 7 (20) | 5 (31) | 2 (11) | 0.208 |
| Plasmapheresis | 7 (20) | 4 (25) | 3 (16) | 0.677 |
| CRRT | 10 (29) | 9 (56) | 1 (5) | 0.002 |

*NA, not applicable; CT, computed tomography; IVIG, intravenous immunoglobulin; CRRT, continuous renal replacement therapy.
†Fisher exact test was used to compare groups.

aspartate aminotransferase, lactate dehydrogenase (LDH), creatinine kinase, and creatinine kinase myocardial b fraction levels were similar for both groups. However, the maximum alkaline phosphatase (ALP) levels during the first week after symptom onset were significantly higher for patients who died than for those who recovered (213 U/L vs. 79 U/L; p=0.017).

## Discussion

We found that SFTS cases occurred throughout South Korea, although the incidence was higher in the southern part of the country. Among the various clinical manifestations, neurologic symptoms (overall and within 7 days after disease onset) were substantially more frequent among the case-patients who died, although GI symptoms and hemorrhagic manifestations (except melena) were more frequent among those who recovered. No effective treatment, including ribavirin, was identified.

The higher incidence of SFTS in the southern part of South Korea was particularly notable for Jeju Province, which is the largest island and the most southern part of South Korea. The prevalence of *H. longicornis* ticks in Jeju Province is among of the highest in South Korea (*9*,*10*); high temperatures are conducive to the survival and breeding of this species (*11*), and Jeju Province has the highest average temperature in South Korea, related to its low latitude (*12*). Furthermore, a study of ticks that were collected during 2011–2012 in South Korea reported that the minimum infection rates for SFTSV in *H. longicornis* ticks were higher in the southern part of the country (*6*).

Although the SFTS case-fatality rate in South Korea (46%) was higher than those that have been reported by using SFTS data from China (6%–30%) (*1*,*13*), it was similar to the rate (55%) that was reported in a retrospective tracing study in Japan (*14*). However, after the introduction of the China surveillance system in 2009, a decreasing trend in

**Table 3.** Laboratory features of hospitalized case-patients with confirmed severe fever with thrombocytopenia syndrome during the first week after onset, by outcome, South Korea, 2013*

| Laboratory tests | Median (range) | | | p value† |
|---|---|---|---|---|
| | Total, n = 32 | Died, n = 14 | Recovered, n = 18 | |
| Platelet count, × $10^9$/L‡ | 38 (15–113) | 34 (15–113) | 47.5 (29–107) | 0.054 |
| Leukocyte count, × $10^9$ cells/L‡ | 1.5 (0.7–3.0) | 1.5 (0.7–3.0) | 1.5 (0.7–2.7) | 0.925 |
| ANC, × $10^6$ cells/L, n = 30‡ | 969 (125–3,292) | 1,042 (380–2,367) | 920 (125–3,292) | 0.637 |
| Hemoglobin, g/L‡ | 12.9 (9.2–16.4) | 12.1 (9.2–15.1) | 13.5 (9.8–16.4) | 0.193 |
| aPTT, s, n = 30 | 54 (35–97) | 60 (35–97) | 54 (36–73) | 0.400 |
| AST, U/L, n = 31 | 242 (63–4,567) | 420 (103–4,567) | 223 (63–2,145) | 0.109 |
| ALT, U/L, n = 31 | 77 (27–1,432) | 156 (28–1,432) | 63 (27–477) | 0.186 |
| Total bilirubin, mg/dL, n = 31 | 0.5 (0.2–4.0) | 0.6 (0.3–4.0) | 0.5 (0.2–1.3) | 0.109 |
| Amylase, U/L, n = 26 | 84 (40–333) | 78 (49–163) | 90 (40–333) | 0.540 |
| Lipase, U/L, n = 20 | 98 (38–692) | 92 (38–369) | 117 (40–692) | 0.370 |
| CK, U/L, n = 21 | 570 (67–4,362) | 428 (158–4,362) | 676 (67–2,760) | 0.651 |
| CK-MB, U/L, n = 18 | 6.9 (0.3–300.0) | 4.8 (1.4–300.0) | 7.6 (0.3–35.0) | 0.762 |
| LDH, U/L, n = 28 | 908 (279–4,564) | 1,799 (279–4,564) | 893 (348–3,920) | 0.260 |
| ALP, U/L, n = 31 | 119 (44–1,586) | 213 (53–1,586) | 79 (44–510) | 0.017 |

*ANC, absolute neutrophil count; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CK, creatinine kinase; CK-MB, creatinine kinase myocardial b fraction; LDH, lactate dehydrogenase; ALP, alkaline phosphatase.
†Mann-Whitney U test was used to compare groups.
‡Minimum value.

the case-fatality rate in that country has been observed (*13*), which may be supported by the increased capability to detect mild SFTS cases. Age was associated with a prognosis of death in our study, and other studies of hospitalized patients in China with SFTS have reported similar findings (*15–17*). However, in our study, the median ages for those who died versus those who survived (73.5 years and 61 years, respectively) tended to be higher than those among the patients in China (62.1–74 years and 52.9–60 years, respectively) (*15–17*). This older age might partially explain why we observed a higher case-fatality rate compared with previous reports. However, we did not find any person-to-person clusters of infection, and found only 1 family cluster, although these patterns have been reported in China (*3*,*18*).

The median time from symptom onset to admission was 4 days and from onset to clinical suspicion was 7 days. This finding indicates that there is a delay in diagnosis and appropriate care, which is likely related to lack of clinical experience with SFTS among physicians in South Korea. Nevertheless, despite the lack of a statistically significant relationship between this time period and death, a delayed diagnosis of SFTS could affect the prognosis of the patients.

The fever stage of SFTS occurs during the first week after disease onset and is characterized by the sudden onset of fever and GI symptoms (*19*); our findings were similar. However, we found that only 22% of those patients had a high fever (≥39°C), compared with 73% of hospitalized SFTS patients in China (*19*). Diarrhea was observed in 63% of patients in our study, which is higher than the 27%–57% rates that were observed in previous studies of hospitalized patients with SFTS (*1*,*19*,*20*). However, 43% of the patients in our study experienced vomiting, which is similar to the 31%–47% rates that were reported in the previous studies (*1*,*19*,*20*).

We found that neurologic symptoms were associated with a greater likelihood of death, and similar findings have been reported in previous studies of hospitalized patients in China (*19*,*20*). Moreover, we observed a rapid progression and increased frequency of neurologic symptoms during the fever stage among the patients who died, suggesting that early neurologic symptoms portend a fatal outcome.

Hemorrhagic manifestations have been linked to fatal outcomes among SFTS patients in China (*19*,*20*). However, we did not observe a similar association, with the exception of melena. Differences between the health care systems in South Korea and China, especially regarding the use of appropriate platelet transfusions, may partially explain the differences regarding hemorrhagic manifestations.

The lowest platelet counts during the first week were not substantively different between those who recovered and those who died. However, one study has reported that low platelet counts on admission or during the entire course were associated with SFTS severity (*20*). Therefore, it is possible that our relatively small number of cases may obscure the association of the lowest platelet counts with death among patients in South Korea.

According to 2 studies in China, elevated LDH levels on admission or during the entire course of illness were significantly associated with a fatal outcome (*15*,*20*). However, we did not observe a significant association between the highest LDH levels during the first week or at the time of admission and death (data not shown). We did observe significantly higher ALP levels during the first week among those who died, although a study of ALP levels on admission reported similar levels between those who died and those who recovered on the basis of multivariate analysis findings (*15*). Additional studies are needed to evaluate the

relationships between a prognosis of death and high levels of LDH or ALP.

Our study has several limitations. First, mild cases of SFTS might have been missed, given that all of our case-patients were hospitalized. Thus, the true case-fatality rate in South Korea is likely lower than our reported value, related to case ascertainment bias. Second, because of the limited number of SFTS cases during 2013, we could not perform multivariate analysis for the prognosis. Therefore, our findings should be interpreted with caution. Third, because of the case series design, we could not analyze the risk factors for SFTS infection.

In conclusion, the clinical symptoms of SFTS in South Korea appear similar to those experienced by hospitalized SFTS patients in China. Older age and early neurologic symptoms were associated with a fatal outcome in studies in both countries. Expansion of SFTS surveillance into the outpatient sector, along with the development and incorporation of an SFTSV antibody test into the case detection algorithm, would detect milder cases and enhance completeness of SFTS case detection in South Korea.

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# Invasive Disease Caused by Nontypeable *Haemophilus influenzae*

**Jeroen D. Langereis, Marien I. de Jonge**

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**Learning Objectives**

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

- Describe recent evidence supporting the emergence of invasive nontypeable *H. influenzae* during the last 2 decades
- Discuss mechanisms that may explain the increasing prevalence of invasive nontypeable *H. influenzae*
- Assess potential strategies to implement effective prevention of invasive nontypeable *H. influenzae*

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The incidence of severe *Haemophilus influenza* infections, such as sepsis and meningitis, has declined substantially since the introduction of the *H. influenzae* serotype b vaccine. However, the *H. influenzae* type b vaccine fails to protect against nontypeable *H. influenzae* strains, which have become increasingly frequent causes of invasive disease, especially among children and the elderly. We summarize recent literature supporting the emergence of invasive nontypeable *H. influenzae* and describe mechanisms that may explain its increasing prevalence over the past 2 decades.

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*Haemophilus influenzae* is an extracellular bacterium that commonly colonizes the upper respiratory tract of healthy humans, who are the bacterium's only known natural reservoir. The *H. influenzae* species is subdivided into 7 groups, including 6 that express distinct serotypes of polysaccharide capsule (a–f) and 1 unencapsulated group termed nontypeable *H. influenzae* (NTHi). NTHi is most frequently associated with mild inflammatory diseases of the human mucosa, including otitis media (OM), sinusitis, and exacerbations of chronic obstructive pulmonary disease (COPD), but it can also cause invasive disease (*1*). The incidence of invasive NTHi (usually defined as isolation of NTHi from a normally sterile site) has increased substantially since the introduction of the *H. influenzae* serotype b (Hib) vaccination in the early 1990s and of the *Streptococcus pneumoniae* polysaccharide conjugate vaccine (PCV) in the early 2000s (*2–5*), but factors contributing to NTHi are poorly understood. We summarize data supporting the emergence of NTHi as an increasingly prominent cause of invasive bacterial disease and propose 4 factors that may be driving its rising prevalence worldwide.

## Methods

We first summarized nationwide surveillance of invasive NTHi disease recorded by the Netherlands Reference Laboratory for Bacterial Meningitis (*6*). Next, on November 12, 2014, we systematically searched the US National Library of Medicine's PubMed database (http://www.ncbi.nlm.nih.gov/pubmed/) by using the search terms "invasive nontypeable *Haemophilus influenzae*" and "invasive non-typeable *Haemophilus influenzae*." We reviewed all papers published during 2000–2014 and summarized all surveillance studies meeting the following criteria: 1) written in English; 2) recording invasive *H. influenzae* cases occurring during the post-Hib vaccine era; 3) spanning ≥4 years; and 4) discriminating among serotype b, non–serotype b, and NTHi strains (Table; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/15-0004-Techapp.pdf). Finally, we described mechanisms that may explain increased prevalence of invasive NTHi infections over the past 2 decades.

## NTHi as Emerging Pathogen Causing Invasive Disease in the Netherlands

Until the mid-1990s, *H. influenzae* serotype b (Hib) was a predominant cause of invasive disease (e.g., pneumonia, sepsis, and meningitis), especially in children. In 1992, a total of 294 (93%) cases with *H. influenzae* isolates that caused sepsis or meningitis in the Netherlands were attributed to Hib alone. The introduction of an effective vaccine in 1993 drastically decreased the incidence of serotype b infections, which, by 1997, represented only 19 (22%) cases with invasive *Haemophilus* isolates (*6*). However, with the near-elimination of invasive disease caused by Hib, the number of recorded invasive NTHi cases increased almost 6-fold during the past 2 decades, from 20 in 1992 to 115 in 2013 (Figure 1). Most (95%) of these NTHi isolates were collected from blood (*6*). NTHi invasion was detected mainly among persons >50 years of age (80%); Hib was found more often in children <5 years of age, who represented 45% of cases, compared with 38% for those >50 years of age (*6*). As with the increased number of invasive NTHi cases, a notable increase in the prevalence of non–serotype b encapsulated *H. influenzae* strains was also observed in the Netherlands (Figure 2), although the number of such cases remains small (*6*).

## NTHi as Emerging Pathogen Causing Invasive Disease Worldwide

Emergence of NTHi as a cause of invasive disease was reported in studies worldwide and was consistently the most prevalent *H. influenzae* that caused invasive disease (Table). In contrast, Hib cases represented <20% of invasive disease. Seven studies showed a clear increase in absolute numbers of invasive NTHi cases or increased incidence rates of invasive NTHi during the study period (online Technical Appendix references *1,7,8,11,15–17*); 4 studies showed no increase (online Technical Appendix references *2,3,5,6*). Large year-to-year differences in overall *H. influenzae* incidence reported by Laupland et al. make it difficult to interpret whether an increase in invasive NTHi incidence occurred (online Technical Appendix reference *6*). Furthermore, a relatively low number of patients (n = 122) over an extended collection period of 7 years complicates the year-to-year analysis in the study by Tsang et al. (online Technical Appendix reference *2*). However, that study showed a significantly higher mean number of NTHi infections during 2004–2006 (12.7 ± 2.5), compared with the mean number during 2000–2003 (5.0 ± 2.6). Bamberger et al. observed no difference in the incidence of invasive NTHi, perhaps explained by the study population, which consisted of children <15 years of age (online Technical Appendix reference *5*). Globally, the average incidence of invasive NTHi is ≈1/100,000 population (*4*,*7*,*8*), a rate similar to that of the Netherlands (*9*).

Whereas Hib predominantly causes bacterial meningitis in healthy children <5 years of age, most invasive NTHi disease is found in very young children (<20 weeks of age) and the elderly (>65 years). In these populations, NTHi develops as pneumonia or bacteremia without apparent focus of infection (online Technical Appendix references *3,6,16,17*). These findings contrast with the widely held view that NTHi infections are mild or asymptomatic. The potential severity of invasive NTHi is illustrated by case fatality rates of 10%–20% (online Technical Appendix references *3,6,16*), similar to case fatality rates for *S. pneumoniae* (*10*).

**Table.** Invasive *Haemophilus influenzae* cases worldwide since introduction of serotype b vaccine*

| Location | Period of strain collection | Surveillance method | Typing method | Changes in NTHi cases or incidence† | Serotyped Hi isolates, no. | Serotype b isolates, % | Non–serotype b isolates, % | NTHi isolates, % | Ref |
|---|---|---|---|---|---|---|---|---|---|
| Canada | 1989–2007 | Active, prospective surveillance | SA | Increased incidence | 1,455 | 20 | 17 | 62 | (*1*) |
| Canada | 2000–2006 | Nationwide surveillance | SA+PCR | No change | 122 | 4 | 39 | 57 | (*2*) |
| Europe | 1996–2006 | European Union Invasive Bacterial Infection Surveillance | SA or PCR | No change | 7,992 | 35 | 9 | 56 | (*3*) |
| Germany | 2001–2004 | Nationwide surveillance | Not reported | NA | 147 | 40 | 14 | 46 | (*4*) |
| Israel‡ | 2003–2012 | Nationwide prospective surveillance | SA | No change | 389 | 26 | 11 | 62 | (*5*) |
| Multiple§ | 2000–2008 | Active population-based surveillance | Not reported | No change | 398 | 6 | 17 | 77 | (*6*) |
| Portugal | 2002–2010 | Laboratory-based passive surveillance | PCR | Increased cases | 144 | 13 | 10 | 77 | (*7*) |
| Slovenia | 2000–2008 | National surveillance | PCR | Increased incidence | 108¶ | 13 | 2 | 85 | (*8*) |
| Spain | 2004–2009 | Nationwide surveillance | PCR | NA | 307 | 5 | 8 | 87 | (*9*) |
| Spain | 2008–2013 | Laboratory-based study | SA | NA | 70 | 1 | 14 | 85 | (*10*) |
| Sweden | 1997–2009 | Retrospective laboratory-based study | PCR | Increased cases or incidence | 268# | 11 | 18 | 71 | (*11*) |
| Taiwan | 1999–2002 | National surveillance | SA | NA | 10 | 20 | 0 | 80 | (*12*) |
| USA, Alaska | 1991–1996 | Active surveillance | SA | NA | 40 | 14 | 31 | 54 | (*13*) |
| USA, Arkansas | 1993–2001 | Retrospective laboratory-based study | SA | NA | 33 | 3 | 6 | 91 | (*14*) |
| USA, Utah | 1998–2008 | Passive surveillance | SA | Increased cases or incidence | 101 | 9 | 49 | 43 | (*15*) |
| USA, Illinois | 1996–2004 | Passive surveillance | SA | Increased incidence | 522 | 15 | 31 | 54 | (*16*) |
| USA | 1999–2008 | Active surveillance | SA | Increased incidence | 4190 | 4 | 26 | 70 | (*17,18*) |

*Hi, *Haemophilus influenzae*; NA, not applicable due to limited sample size (<100 isolates) or lack of year-to-year data; NTHi, nontypeable *Haemophilus influenzae*; Ref, reference (see online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/15-0004-Techapp.pdf); SA, slide agglutination; SA+PCR, slide agglutination positive isolates confirmed by PCR.
†Increased cases = increase in number of NTHi cases in patients ≥1 years of age; Increased incidence = increase in NTHi incidence rate in patients ≥1 year of age; No change = no difference in number or incidence rate of NTHi cases.
‡Pediatric cases (<15 years of age) only.
§Australia, Canada, and Denmark.
¶PCR-typed isolates from post-Hib vaccination era only.
#PCR-typed isolates only.

## Emergence of Capsulated, Non–Serotype b *Haemophilus influenzae* Strains

In addition to the increased incidence of NTHi infections, an increased number of invasive infections caused by encapsulated non–serotype b *H. influenzae* strains, especially Hie and Hif, has been observed in the Netherlands during the past 2 decades (Figure 2). This trend has been confirmed in multiple independent studies (online Technical Appendix references *3,11,17*), although the increases are not as large as those observed for NTHi. In Europe, the incidence of non–serotype b *H. influenzae* capsulated strains was 690 (9%) of 7,992 isolates; 500 (72.5%) of the 690 isolates were Hif, and 143 (20.7%) were Hie (online Technical Appendix reference *3*). This distribution of non–serotype b *H. influenzae* encapsulated serotypes causing invasive disease was similar in other parts of the world, except for specific

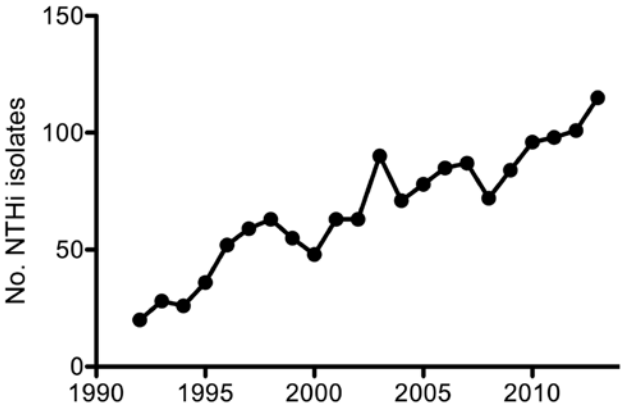


**Figure 1.** Number of recorded nontypeable *Haemophilus influenzae* (NTHi) isolates from blood or cerebrospinal fluid in the Netherlands, by year, 1992–2013. Adapted from (*6*).

ethnic groups where Hia is most prevalent (*11–13*). The epidemiology and clinical manifestations of invasive Hie and Hif strains are similar to that of NTHi and mostly occur as pneumonia or bacteremia in the elderly (*14*). In contrast, invasive Hia infections are more similar to Hib infections than to NTHi infections. Hia infections occurred mainly in young children and frequently as meningitis (*12*,*13*). The apparent similarity between Hia and Hib might be attributed to similarities in capsule structure; both contain a neutral sugar, an alcohol (ribitol), and a phosphodiester (*15*).

## Possible Explanations for Emergence of Nontypeable *Haemophilus influenzae*

Although numbers of NTHi cases are increasing, underlying mechanisms for the increase are yet to be determined. We offer 4 possible explanations for the emergence of NTHi as a pathogen causing invasive disease.

### 1) Vaccine-Mediated Strain Replacement

The success of the Hib vaccine and PCV is attributed to the strong immunogenic properties of polysaccharide conjugate formulations. However, protection against *H. influenzae* is limited to serotype b, and the possibility exists that another disease, caused by other *H. influenzae* strains against which vaccines offer no protection, may replace Hib. In fact, already in 1997, Marc Lipsitch anticipated the possibility of strain replacement with the introduction of the Hib vaccine (*16*). The significance of strain replacement remains controversial: some clinical studies highlight its potential danger (*2*,*4*); others fail to observe it altogether (*8*,*17*). Besides the introduction of the Hib vaccination, introduction of the PCV has also been proposed as contributing to *H. influenzae* strain replacement. Multiple studies show substantial increases in NTHi nasopharyngeal colonization (*3*,*5*) and in percentage of OM cases caused by NTHi in PCV-vaccinated persons (*18–20*). This increased nasopharyngeal carriage of NTHi in PCV-vaccinated children might increase transmission to groups susceptible to invasive NTHi disease, such as the elderly, and might thereby contribute to the emergence of invasive NTHi disease. Strain replacement during colonization of persons >65 years of age appears to lack investigation, possibly because of the relatively low percentage of nasopharyngeal carriage in this age group. However, NTHi carriage rates in parents of PCV-vaccinated children increased from 23% prevaccination to 40% postvaccination (*5*), indicating that carriage of NTHi has increased in healthy adults, possibly because of increased transmission from PCV-vaccinated children. Increased NTHi carriage might contribute to the increased number of invasive NTHi disease cases recorded during the past 20 years.

### 2) Improved Bacterial Detection and Serotyping

Whether the emergence of NTHi as a cause of invasive disease indicates an actual increase in the number of cases or results from improved detection and serotyping is difficult to assess. Bacterial culture is the gold standard for *H. influenzae* detection. However, a major disadvantage of culture is that multiple days are needed to isolate bacteria and confirm culture identity. Therefore, rapid and more sensitive real-time PCR (rtPCR) assays have been developed to shorten the time needed for identification. Several rtPCR assays that target different *H. influenzae* genes have been developed and are more sensitive for detecting the *hpd* gene than for detecting genes *ompP2* or *bexA* (detection of capsulated *H. influenzae* strains only) (*21*). Despite evidence that rtPCR-based assays provide improved detection of *H. influenzae*, all studies we summarize use bacterial culture as the detection method (Table).

Slide agglutination is the gold standard for serotyping *H. influenzae* in most laboratories, although this technique is prone to misinterpretation because of nonspecific agglutination, cross-reactions, or autoagglutination. The transition from slide-agglutination to PCR-based methods that detect capsule locus genes, such as *bexA* or *bexB*, has substantially improved the accuracy of serotyping results. For instance, Kastrin et al. recently showed that 80 isolates originally serotyped as NTHi were detected as unencapsulated by PCR, but 12 (11% of total isolates) of 28 isolates reported as capsulated by slide agglutination were shown by PCR to harbor no functional capsule genes (*7*). On the basis of PCR results, 5%–20% of strains typed by slide agglutination were mistyped as encapsulated (*7*,*22*,*23*). Although PCR detection methods apparently detect more invasive NTHi isolates than does slide agglutination, the increased detection by PCR does not explain the year-to-year increase in number of invasive NTHi cases (Table) because, within each study, similar typing techniques were

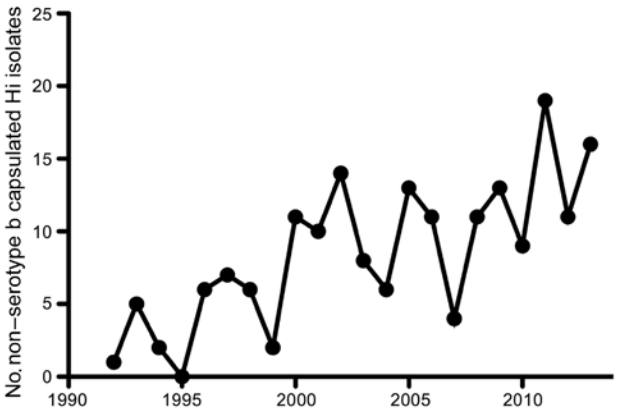


**Figure 2.** Number of recorded non–serotype b capsulated *Haemophilus influenzae* (Hi) isolates from blood or cerebrospinal fluid in the Netherlands, by year, 1992–2013. Adapted from (*6*).

used throughout the study period. However, the number of invasive NTHi cases likely is underrepresented in studies that use slide agglutination for detection.

### 3) Increased Virulence of NTHi Strains

Increased bacterial virulence as a consequence of the acquisition of novel virulence factors might also contribute to invasive NTHi disease. The natural genetic competence of NTHi enables the exchange of large pieces of DNA between strains at a high frequency (*24*), a process that supports acquisition of novel virulence factors.

Because invasive infection and death of host do not enhance transmissibility of virulent NTHi, the evolutionary basis for these genetic changes may lie in fitness advantages during nasopharyngeal colonization, a theory supported by recent studies. For instance, we have shown that NTHi isolates collected from middle ear fluid of children with OM exhibited increased resistance to complement-mediated killing compared with colonizing NTHi isolates from the nasopharynx (*25*), but we found that colonizing and OM-causing NTHi strains with a similar multilocus sequence type collected from the same patient showed no difference in complement resistance (*26*). This similarity in complement resistance for NTHi strains with a similar multilocus sequence type indicates that NTHi strains had already acquired mechanisms that increased resistance to complement-mediated killing during colonization and retained them during translocation to the middle ear cavity. These observations were corroborated by a later study that showed that most of the phase-variable genes known to modulate resistance to complement-mediated killing were regulated similarly for colonizing and OM-causing NTHi strains within the same patient (*27*).

Limited data are available on the mechanisms that underlie increased NTHi virulence in patients with invasive disease. Recently, Bajanca-Lavado et al. showed that an NTHi strain that caused endocarditis appeared highly virulent because of the expression of a second copy of the IgA protease gene (*iga*B) combined with a strong resistance to complement-mediated killing (*28*). We have shown that NTHi strains collected from patients with invasive disease more frequently incorporate galactose to heptose III in the lipooligosaccharide; this modification decreases binding of IgM and thereby increases resistance to complement-mediated killing (*29*). A study by Hällstrom et al. showed a correlation between complement resistance and disease severity but no difference in complement resistance between invasive and colonizing NTHi strains (*30*). This lack of difference in complement resistance between invasive and colonizing NTHi strains might be explained by the large proportion (41%) of patients with immune deficiencies in the invasive group, therefore potentially reducing the immunologic pressure for NTHi strains to maintain complement resistance in the bloodstream.

That the type b capsule protects *H. influenzae* from the bactericidal activity of the complement system and that this protection contributes to its invasive character are generally accepted ideas. Zwahlen et al. reported that capsule transformants showed dramatic differences in virulence (*31*). Although all capsule types were able to colonize the nasopharynx of rats, bacteremia was detected only in animals challenged with serotypes a and b and with a single animal serotype, f. The highest bacterial load was found among animals infected with serotype b. Therefore, losing the protective capsule would be expected to render *H. influenzae* unable to cause invasive disease. However, recent whole genome sequencing results showed that a few invasive NTHi isolates had a multilocus sequence type usually associated with serotype b strains (*32*). In these NTHi isolates, lack of capsule expression was related to the deletion of the *bexA* gene, whereas the remaining capsule locus was similar to that of the corresponding capsulated isolates. However, the lack of a capsule did not abrogate the ability of these particular NTHi strains to cause invasive disease, indicating that other factors besides the capsule of Hib strains contribute to invasiveness. For example, Fleury et al. found that a Hib- and Hif-specific lipoprotein PH was able to bind human factor H, resulting in increased resistance to complement-mediated killing (*33*). Identification of other genetic factors might partly explain why NTHi is found to cause invasive disease.

### 4) Demographic Changes

The epidemiology of invasive *H. influenzae* disease has changed dramatically over the past 20 years (*2*,*7*). Instead of being mainly a pediatric disease caused by Hib, formerly rare capsular serotypes (mostly Hia and Hif) and NTHi cause most of invasive *H. influenzae* cases, especially

among the elderly (Table). For instance, in the United States, 78% of invasive *H. influenzae* cases among adults >65 years of age were attributed to NTHi, with an even higher frequency (89%) among those >85 years of age (*34*).

Reasons for this apparent increase in susceptibility to invasive NTHi infections in the elderly are unknown, but the immunologic status of the host is believed to play a role. Coexisting conditions or risk factors such as coronary artery disease, congestive heart failure, and smoking were more common in patients with invasive disease compared with the general population (*34*). The number of patients with COPD, the third leading cause of death worldwide (*35*), is increasing. NTHi is often found colonizing the lungs of patients with COPD, and the increased number of patients with COPD might contribute to the increased incidence of invasive NTHi cases. Serum IgG levels to *H. influenzae* protein D showed a tendency to decline with age but were even lower in adults with coexisting conditions such as COPD, cancer, chronic renal failure, or diabetes, compared with age-matched healthy persons (*36*). The absence of naturally acquired antibodies against protein D, a highly conserved antigen, may contribute to increased susceptibility to invasive NTHi disease. However, invasive NTHi infections are found not only in persons with immunocompromising conditions (e.g., chronic lymphatic leukemia or multiple myeloma) or coexisting conditions (e.g., COPD, diabetes, or cardiovascular diseases) but in almost half of cases in persons who were otherwise in good health (online Technical Appendix references *6,11*).

Recently, several groups have found that binding of IgM to the bacterial surface might play a role in the innate defense against NTHi infections (*25*,*29*,*37*). This finding is corroborated by a clinical study in which Micol et al. showed that patients with hyper-IgM syndrome were less susceptible to NTHi colonization, a finding that emphasizes the role of IgM in the immune defense against this pathogen (*38*). The percentages of IgM-producing CD27+ memory B cells in the peripheral blood of children are low but increases to almost 20% in adults and declines again in the elderly (*39*). These findings correspond with levels of susceptibility to bacterial infections such as NTHi in young children and the elderly. Studies examining serum immunoglobulin levels in patients with invasive NTHi disease compared with those of healthy age-matched patients could help address the question of whether a diminished protective immunoglobulin level in the elderly contributes to susceptibility to invasive NTHi disease.

Besides impaired humoral immunity, diminished cellular immunity has been described in the elderly. Evidence exists for a broad, age-related alteration in the development and function of lymphocytes, monocytes, macrophages, and neutrophils (*40*), although specific effects of these changes on susceptibility to invasive NTHi infections have not been investigated in detail. Recently, we showed that neutrophils efficiently phagocytose and kill opsonized NTHi bacteria (*29*), but decreased neutrophil phagocytic capacity among the elderly may impair this host defense and contribute to poorer clinical outcomes during NTHi infection.

## Conclusions

From examination of the available literature, we conclude that invasive NTHi disease is emerging worldwide and demands implementation of effective prevention. Development of vaccines against NTHi is considered paramount because this pathogen is also often found to cause pneumonia in patients with COPD and OM in children. However, development of an effective vaccine for risk groups demands knowledge about factors that contribute to the emergence of invasive NTHi disease. Age and coexisting conditions are likely predisposing factors for invasive NTHi infections. Also, increased NTHi colonization in children might contribute to increased transmission to persons susceptible to developing invasive NTHi disease. In view of these factors, broad vaccination strategies for the general public could be effective by decreasing transmission, bolstering herd immunity, and protecting potentially susceptible persons.

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# Cluster of *Cryptococcus neoformans* Infections in Intensive Care Unit, Arkansas, USA, 2013

**Snigdha Vallabhaneni, Dirk Haselow, Spencer Lloyd, Shawn Lockhart, Heather Moulton-Meissner, Laura Lester, Gary Wheeler, Linda Gladden, Kelley Garner, Gordana Derado, Benjamin Park, Julie R. Harris**

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**Release date: September 16, 2015; Expiration date: September 16, 2016**

**Learning Objectives**

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

- Assess the epidemiology and clinical presentation of *C. neoformans* infection
- Distinguish the clinical presentation and outcomes of *C. neoformans* infection in the current study
- Analyze exposure variables associated with *C. neoformans* infection in the current study
- Identify clinical variables associated with *C. neoformans* infection in the current study

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We investigated an unusual cluster of 6 patients with *Cryptococcus neoformans* infection at a community hospital in Arkansas during April–December 2013, to determine source of infection. Four patients had bloodstream infection and 2 had respiratory infection; 3 infections occurred within a 10-day period. Five patients had been admitted to the intensive care unit (ICU) with diagnoses other than cryptococcosis; none

had HIV infection, and 1 patient had a history of organ transplantation. We then conducted a retrospective cohort study of all patients admitted to the ICU during April–December 2013 to determine risk factors for cryptococcosis. Four patients with *C. neoformans* infection had received a short course of steroids; this short-term use was associated with increased risk for cryptococcosis (rate ratio 19.1; 95% CI 2.1–170.0; p<0.01). Although long-term use of steroids is a known risk factor for cryptococcosis, the relationship between short-term steroid use and disease warrants further study.

*Cryptococcus neoformans* is an encapsulated yeast found in soil throughout the world, particularly in soil contaminated with pigeon guano (*1*). Persons become infected by inhaling fungal spores (*2*), and the infection is usually asymptomatic. In some persons, latent infections can be established in the lungs and hilar lymph nodes (*3*). Cryptococcal disease typically manifests when latent infection is reactivated after a person becomes immunosuppressed (e.g., receives long-term steroids or immunosuppressive medications for an organ transplant or has advanced HIV infection) (*4*). Meningitis and pneumonia are the most common manifestations, and bloodstream infection (BSI) occurs far less frequently (*5*). Because cryptococcosis rarely results from acute fungal exposure and because person-to-person transmission is exceedingly uncommon (*6*,*7*), clusters of patients with this disease are unusual.

Hospital A is a community hospital in northwestern Arkansas, USA, with ≈300 beds, 38 of which are intensive care unit (ICU) beds. *C. neoformans* was isolated from 6 patients during 2013: 4 patients had *C. neoformans* BSI, and 2 had bronchoalveolar lavage (BAL) specimens yielding *C. neoformans.* Three of the 4 *C. neoformans* BSI cases occurred within a 10-day period in April–May 2013. Although *C. neoformans* infection is not a reportable condition in Arkansas, an astute infection control practitioner (ICP) at hospital A noted the unusual number of *C. neoformans* BSIs in a short period and contacted the Arkansas Department of Health. Preliminary investigation revealed that most case-patients had been admitted to the hospital A ICU, which prompted concern that the ICU might be the source of acute cryptococcal infection. All case-patients died, most within days of their positive *C. neoformans* culture. This unusual set of circumstances—clustering in time of an uncommon disease among patients with exposure to a single hospital unit—combined with the high death rate led to an on-site investigation to further characterize clinical illness among patients in this cluster and identify possible sources of and risk factors for infection.

## Methods

A case was defined as culture-confirmed *C. neoformans* infection in an inpatient admitted to hospital A during 2013. We reviewed microbiology records at hospital A for 1992–2013 to identify all cases and establish a background rate for positive cryptococcal cultures at hospital A. We telephoned microbiology staff and ICPs at surrounding hospitals to inquire about any recent changes noted in rates of isolation of *Cryptococcus* spp.

Case-patients' medical records were reviewed, and data were abstracted by using a standardized case report form that included demographic and clinical information. Case-patients' family members (or case-patients, if alive) were interviewed by using a standardized questionnaire to identify potential exposures to *Cryptococcus.* Questions included whether patient had contact with pigeons or other birds and whether case-patients had engaged in any common activities in the community that could have resulted in a common acute exposure to *C. neoformans* before hospital admission. We interviewed laboratory managers and technicians at hospital A to assess specimen collection and processing methods and to identify any potential sources for contamination of specimens with *C. neoformans*.

To assess the possibility of acute, nosocomially acquired *C. neoformans* infection, we took several steps. Case-patient charts were reviewed for commonalities in physical location, procedures, and providers. ICU staff, hemodialysis staff, and ICPs at hospital A were interviewed to gain an understanding of how patients are cared for in the ICU, dialysis procedures, and infection control practices. We also asked hospital leadership about any known history of illegal activity or recent disciplinary action of ICU staff members. We asked facilities management staff about any bird habitats at the hospital and recent construction activity. Because *C. neoformans* thrives in bird guano, we also interviewed all ICU staff members about contact with birds and sampled the hands using Handi-wipes (*8*) and homes using Sponge-Sticks (3M Co., St. Paul, MN, USA) and vacuum filter socks (X-Cell 100 Dust Sampling Sock, Midwest Filtration Co, Cincinnati, OH, USA) (*9*) of ICU staff members who reported substantial bird exposure and had contact with case-patients. Environmental sampling was conducted in the ICU with Sponge-Stick swabs.

Finally, we conducted a retrospective cohort study to identify factors associated with cryptococcosis. Patients admitted to the hospital A ICU during April 1–December 31, 2013 (the period during which the cases occurred), were included. Patients were identified by querying the electronic medical record database. Data extracted from the medical record included length of stay in the ICU, receipt of glucocorticoids in the ICU, type of steroid if one was administered, and whether the patient was cared for by a specific respiratory therapist. For the purposes of the cohort analysis, only case-patients who were admitted to the ICU were included (n = 5). Poisson regression models were fit to the data to examine the relationship between the

potential risk factors and cryptococcosis and to estimate the rate ratios between different exposure groups.

Clinical isolates were confirmed as *C. neoformans* by melanin production on L-DOPA media and by lack of growth after inoculation on canavanine-glycine-bromothymol blue media (*10*). Isolates were subtyped by using multilocus sequence typing (MLST). The *URA5, IGS1, CAP59, LAC1, GPD1, PLB1,* and *SOD1* gene fragments were amplified as described (*11*) for all isolates. Allele numbers and sequence types were determined by using the online *C. neoformans* MLST database (*12*). Environmental samples were processed and plated onto birdseed benomyl agar, incubated at 35°C, and observed for growth at 4, 7, and 14 days.

## Results

### Case-Patient Descriptions

We identified 6 cases of *C. neoformans* infection at hospital A during 2013: 4 case-patients had BSI, and 2 case-patients had respiratory specimens (obtained from BAL) that yielded *C. neoformans.* One of the patients with a BSI also had *C. neoformans* isolated from cerebrospinal fluid (CSF) and urine specimens. The positive cultures were obtained from case-patient 1 on April 6 (respiratory specimen), from case-patient 2 on April 29 (blood), from case-patient 3 on May 1 (blood), from case-patient 4 on May 9 (blood, CSF, urine), from case-patient 5 on June 12 (respiratory specimen), and from case-patient 6 on December 31 (blood).

Case-patient ages ranged from 51 to 82 years; 3 were men (Table, http://wwwnc.cdc.gov/EID/article/21/10/15-0249-T1.htm). Underlying chronic medical conditions included diabetes in 3 patients, asthma/emphysema in 2 patients, and malignancy in 2 patients; 1 patient had metastatic lung cancer, 1 had chronic lymphocytic leukemia, 1 had chronic renal disease requiring hemodialysis, and 1 had received a kidney transplant. None were known to be infected with HIV.

Case-patients were admitted with a variety of diagnoses or symptoms: 3 (50%) had pneumonia with sepsis or respiratory failure, and 1 (17%) each had severe anemia and acute renal failure; nausea, vomiting, and confusion; and chest pain. Three case-patients were directly admitted to the ICU from the emergency department, 2 case-patients were admitted to the ICU 24–48 hours after hospital admission because of new clinical deterioration, and 1 case-patient was never admitted to the ICU. Cultures yielding *C. neoformans* were obtained 1–45 days after hospital admission. In 3 of the 4 cases of *C. neoformans* BSI, case-patients had at least 1 negative blood culture before *C. neoformans* BSI was diagnosed, indicating that the disease likely developed while the patient was in the hospital. All case-patients died; 5 died within 5 days after collection of their clinical sample that yielded *C. neoformans*, 4 before culture results were available. One case-patient, a kidney transplant recipient who exhibited nausea, vomiting, and confusion, received a diagnosis of cryptococcal meningitis on the basis of a positive CSF culture and BSI and did not require admission to the ICU. He survived to hospital discharge but died several months later of unrelated causes. Two case-patients had a serum cryptococcal antigen (CrAg) test: case-patient 1, who had respiratory *C. neoformans* infection and whose CrAg test result was negative, and case-patient 4, who had meningitis and a BSI, whose CrAg test result was positive. No autopsies were performed.

### Background Rates of *Cryptococcus* spp.

Review of microbiology laboratory records at hospital A identified a median of 2 patients (range 0–8) per year with positive *C. neoformans* cultures during 1992–2012; most were from respiratory samples. Only 4 blood cultures yielded *C. neoformans* during this time: 1 in 2004, 2 in 2007 (February and August), and 1 in 2008 (Figure). In contrast, 4 BSIs occurred during 2013, with 3 occurring within 10 days of each other. Laboratory staff and ICPs at 4 surrounding hospitals had reported no increase in the

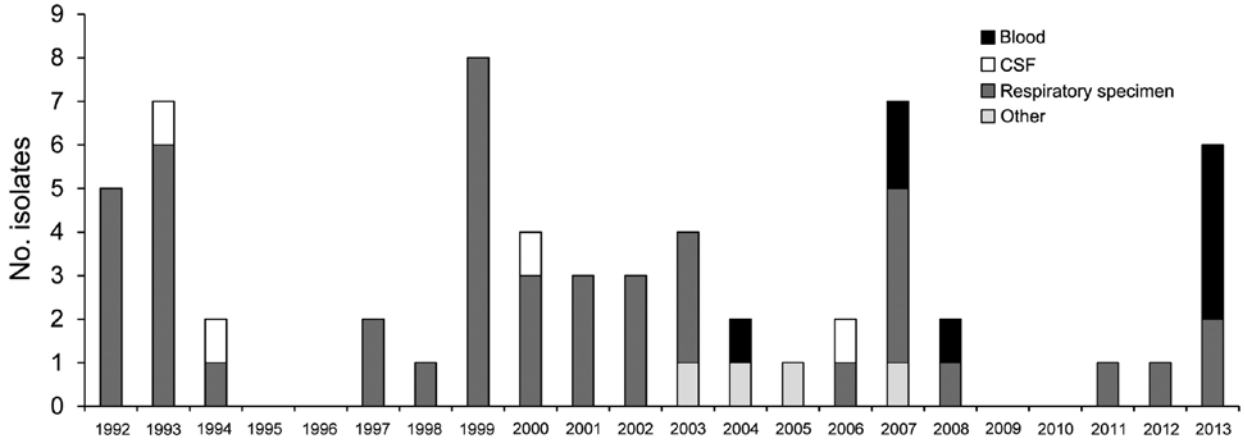


**Figure.** Culture-confirmed *Cryptococcus* isolates at hospital A, Arkansas, USA, 1992–2013. CSF, cerebrospinal fluid.

number of *C. neoformans* isolates since January 2013, indicating that the increase in cases, especially BSI cases, was only occurring at hospital A.

### Evaluation of Specimen Procurement and Processing

Detailed assessment of specimen procurement and hospital laboratory processing methods did not reveal any evidence of laboratory contamination to suggest that this was a pseudo-outbreak. Specimens from case-patients were procured by different personnel in different parts of the hospital (emergency room, medical floor) on different days. Blood cultures were processed in a closed system (BACTEC; Becton Dickinson, Franklin Lakes, NJ, USA), and in-laboratory location, growth media, and method of processing BAL and blood cultures differed.

### Evaluation for Common Exposures

Interviews with family members of case-patients did not reveal common community exposures. Case-patients lived in 4 different towns and did not work or attend religious services or social events in the same places. None of the case-patients had any recent exposure to birds, including pigeons.

Five of 6 case-patients were admitted to the ICU before their culture was positive: case-patients 1, 2, and 3 were in adjacent ICU rooms during April 6–April 14, 2013. All 5 case-patients in the ICU were also cared for by the same ICU physician; 4 of 5 had been cared for by 1 respiratory therapist (RT). No other recognized commonalities were found between case-patients in terms of procedures, devices (including hemodialysis machines and ventilators), or personnel involved in their care.

We interviewed 112 hospital staff members who might have had contact with ICU patients; 8 reported contact with birds. Types of contact included hunting pheasants, feeding backyard chickens, and maintaining outdoor bird feeders. One RT reported having 2 pet cockatiels at home with whom she spent up to 2 hours each day. The RT had cared for 4 of the 5 case-patients who were admitted to the ICU. Among the 7 other staff members with any bird contact, 2 persons, a pharmacist and an x-ray technician, had come into contact with 4 of 5 case-patients in the ICU. Sampling of hands of these 3 health care workers and a vacuum sample of the cockatiel aviary (a sunroom in the house where the birds were kept) belonging to the RT did not yield *C. neoformans.* Hospital leadership indicated that there was no concern about illegal activity or recent disciplinary action against any ICU staff members.

Interviews with facilities management staff revealed that the ICU roof had been leaking for ≈3 years in several locations, including the time case-patients 1–3 were in the ICU, before it was replaced during April 22–May 19, 2013. Notably, staff reported that the roof had been a roost for pigeons approximately 6 years earlier, and the hospital had undertaken pigeon exclusion measures at that time. Environmental swab samples taken in July 2013, ≈8 weeks after the roof replacement was completed, from the ICU rooms where case-patients 1–3 had overlapping stays did not yield *C. neoformans*. Culture of the external air filter leading into the ICU HVAC system also did not yield *C. neoformans.*

### Cohort Study to Assess Risk Factors for Cryptococcosis

A total of 1,606 patients, including 5 of 6 case-patients who had *C. neoformans* infection, were admitted to the hospital A ICU during April 1–December 31, 2013. The remaining case-patient, who had received a renal transplant and exhibited cryptococcal meningitis and cryptococcal BSI, was not admitted to the ICU and was therefore not included in this analysis.

Overall, 125 (7.8%) of the 1,606 patients admitted to the ICU during this period received some type of steroid in the ICU. Four (80%) of the 5 *C. neoformans* case-patients admitted to the ICU received steroids in the ICU before their positive culture: 2 received hydrocortisone for treatment of refractory septic shock (1 for 4 days and another for 11 days); 1 received methylprednisolone for 1 day for tumor lysis syndrome followed by an oral prednisone taper over 21 days (100 mg daily for 3 days, 80 mg daily for 3 days, 40 mg daily for 3 days, and 20 mg daily for 12 days); and 1 received methylprednisolone (5 days) and hydrocortisone (8 days) for treatment of refractory septic shock and ongoing respiratory failure. None of these patients had been receiving steroid treatment before hospital admission. Approximately 8% (121/1,601) of patients without cryptococcosis received steroids in the ICU: 7 received oral prednisone, 22 received hydrocortisone, 73 received methylprednisolone, and 19 received dexamethasone. The rate of cryptococcosis was 40.1 cases per 10,000 person-days in the ICU among persons receiving steroids, compared with 2.1 cases per 10,000 person-days in the ICU among persons not receiving steroids (rate ratio 19.1; 95% CI 2.1–171.1; p = 0.008). Exposure to the RT was also assessed as a risk factor for cryptococcosis, but no significant difference was found.

### Multilocus Sequence Typing (MLST) of Clinical Isolates

Clinical isolates from all 6 case-patients were confirmed at CDC as *C. neoformans* with 3 separate MLST patterns. Isolates from case-patients 1 and 3 had MLST patterns that were indistinguishable from each other, isolates from case-patients 2 and 4 shared a second MLST pattern, and isolates from case-patients 5 and 6 had a third MLST pattern distinct from the other 2 patterns.

## Discussion

We investigated 6 cases of cryptococcosis that occurred in 2013 in a community hospital ICU. For most of the case-patients, the disease appears to have developed while they

were in the ICU after admission for other diagnoses. The patients experienced a fulminant clinical course after the diagnosis of cryptococcosis and died soon thereafter. There was no identifiable point source for the infections in the hospital or the community. Receipt of short-term steroids in the ICU was significantly associated with cryptococcosis in this cohort.

The cluster was characterized by several atypical clinical features. First, active cryptococcal disease is usually associated with HIV infection or organ transplant–associated immunosuppression. Five of 6 patients in this cluster were non–HIV-infected and nontransplant patients (NHNT); however, each did have other predisposing conditions, including chronic renal failure, chronic lung disease, hematologic malignancies, and other malignancies that might have put them at risk for cryptococcal disease (*13*). Second, *C. neoformans* BSI is extremely uncommon, especially among NHNT patients (*14*,*15*), yet 4 case-patients had blood cultures yielding *C. neoformans;* only 1 case-patient (the renal transplant recipient) had meningitis, a more typical manifestation of this disease. Next, acute respiratory failure and overwhelming sepsis, as experienced by 5 patients in this cluster, are atypical manifestations of cryptococcal disease (*16*); cryptococcosis is generally a subacute infection with insidious onset of nonspecific symptoms (*5*), especially among NHNT patients, who may have prolonged symptoms before diagnosis (*17*). In contrast, the 5 NHNT patients in this cluster had a relatively short duration of symptoms and experienced respiratory failure, septic shock, or both and died within days of their positive culture.

Because *C. neoformans* infections rarely result from acute fungal exposure, and because person-to-person transmission of cryptococcosis—if it exists at all—is exceedingly uncommon (*6*,*7*), focal clusters or outbreaks of cryptococcosis are not expected and, to our knowledge, have not previously been reported. Disease usually results from reactivation of latent infection in immunosuppressed hosts; reactivation in 1 host is an independent event that is not necessarily linked to reactivation in another host. The cases we investigated were clustered in space (ICU) and time (2013, with 3 cases occurring with 10 days of each other in late April through early May). The atypical patients and the unusual clinical manifestations involved in this cluster may be an indication that the source or mechanisms of infection and disease, though not identified during the investigation, were not typical for cryptococcosis. If this were a chance clustering of independent occurrences of reactivation of latent cryptococcal infection, we would have expected to see more patients who fit the typical risk profile and have more common manifestations of the disease, and all would not have occurred at a single hospital.

We searched for a point source in both the community and hospital settings. There has been no precedent for *C. neoformans* being found in the hospital environment or being transmitted from health care worker hands, but we investigated these possibilities because they have been implicated in outbreaks with other organisms. We conducted a thorough investigation to identify any hospital sources of *Cryptococcus* spp. but did not find a hospital source through environmental assessment. However, this outcome was limited by the fact that samples were taken ≈12 weeks after the first 3 cases occurred. The meaning of the 3 different MLST patterns among the 6 case-patient isolates is unclear. This finding may be consistent either with a single point source containing multiple strains of *Cryptococcus* spp. (as demonstrated previously) (*17*) or a different, non–point-source cause of infection.

Although nearly two thirds of patients who seek treatment with cryptococcal disease have advanced HIV infection or have received an organ transplant (*3*), long-term oral steroid use is also a known risk factor for cryptococcal disease. In a study of cryptococcal disease among NHNT patients, the median daily dose of prednisone or prednisone-equivalent that patients were receiving was 20 mg, and the median duration of immunosuppression before cryptococcal disease was 7 months (*15*). Although use of steroids in the treatment of sepsis is generally not favored, steroids may be used for a short term in cases of septic shock not responsive to other interventions. A single 10-day tapering course of hydrocortisone used to treat refractory septic shock, as was administered to some of these case-patients, is roughly equivalent to getting 20 mg of prednisone for 15–20 days. Although cases of cryptococcal disease developing in patients receiving low-dose and short-term oral corticosteroids have been reported (*18*), clusters of cryptococcal disease in an ICU setting after receipt of short-term steroids have not been previously described. A combination of multiple chronic underlying conditions, including diabetes, renal failure, and malignancy, which are also known but less frequently associated risk factors for cryptococcosis, and short-term steroid use in the ICU may have contributed to reactivation disease or dissemination of acute infection acquired from an unknown source in the community or the hospital. Further research should be conducted to better understand the relationship between short-term steroid use and the risk for opportunistic infections, including cryptococcal disease.

This investigation has several limitations. First, we considered that all patients with positive *C. neoformans* cultures had cryptococcosis. However, the manifestations were unusual, raising the possibility that some or all case-patients might not have had true cryptococcal disease. Unfortunately, serum CrAg testing results, pathologic specimens, and autopsy findings, all of which might have helped definitively determine if true cryptococcal disease was present, were not available for most case-patients.

Second, although we cannot definitively rule out the possibility of a pseudo-outbreak from laboratory contamination of specimens, *C. neoformans*, unlike environmental molds, is not typically a laboratory contaminant. Thus, we believed that laboratory contamination was a less likely explanation for the cluster. The findings of *C. neoformans* in different types of specimens also made laboratory contamination less likely.

Third, we did not investigate the possibility of a common contaminated intravenous medication as a source for these infection and therefore cannot rule out this explanation as a cause of the outbreak. Again, given the different sites of infection (both respiratory and bloodstream) among case-patients, a common-source contaminated intravenous fluid also appeared unlikely.

Finally, we were only able to look at a limited number of co-variates in the cohort study because of the reliance on an automated query of the electronic database. Individual chart review to determine severity of illness or underlying conditions was not possible for all 1,600 patients in the ICU. Therefore, we could not control for differences in reasons for admission to the ICU and severity of underlying illness.

Although we did not find a point source for infections in the hospital or community, we found that short-term steroids used in the ICU were associated with case status. To clarify whether this association between short-term steroid use and cryptococcosis is generalizable, similar studies examining rates and duration of ICU steroid use and cryptococcosis should be conducted at other hospitals. *C. neoformans* infection may need to be included in the differential diagnosis of a patient receiving short-term steroids in the ICU setting.

We recommended heightened vigilance for cryptococcal infection among ICU patients at hospital A, especially those receiving steroid treatment. We also asked that physicians carefully assess the need for steroid use in patients admitted to the ICU and weigh the risk for possible cryptococcal infection against the benefits of steroid use in each patient's case.

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# Electronic Public Health Registry of Extensively Drug-Resistant Organisms, Illinois, USA

**William E. Trick, Michael Y. Lin, Robynn Cheng-Leidig, Mary Driscoll, Angela S. Tang, Wei Gao, Erica Runningdeer, M. Allison Arwady, Robert A. Weinstein**

In response to clusters of carbapenem-resistant *Enterobacteriaceae* (CRE) in Illinois, USA, the Illinois Department of Public Health and the Centers for Disease Control and Prevention Chicago Prevention Epicenter launched a statewide Web-based registry designed for bidirectional data exchange among health care facilities. CRE occurrences are entered and searchable in the system, enabling interfacility communication of patient information. For rapid notification of facilities, admission feeds are automated. During the first 12 months of implementation (November 1, 2013–October 31, 2014), 1,557 CRE reports (≈4.3/day) were submitted from 115 acute care hospitals, 5 long-term acute care hospitals, 46 long-term care facilities, and 7 reference laboratories. Guided by a state and local public health task force of infection prevention specialists and microbiologists and a nonprofit informatics entity, Illinois Department of Public Health deployed a statewide registry of extensively drug-resistant organisms. The legal, technical, and collaborative underpinnings of the system enable rapid incorporation of other emerging organisms.

The emergence of extensively drug-resistant organisms (XDROs) is a major public health problem because few or no effective antimicrobial drugs are available to treat infections caused by these bacteria (*1*). In the United States, carbapenem-resistant *Enterobacteriaceae* (CRE) are XDROs considered high priority for control (*2–4*), and regional clusters have been detected in Illinois (*5*,*6*) and elsewhere (*7*). Control of drug-resistant bacteria is possible (*8*,*9*) but requires a coordinated regional effort across the spectrum of health care facilities (*10*,*11*). Failure to control spread of antimicrobial drug–resistant bacteria hinders medical care at a growing number of facilities by creating hazardous opportunities for untreatable infections during aggressive medical interventions (*12*), such as immunosuppressive therapies and device insertions, or during common endoscopic procedures (*2*,*13*).

To combat CRE, the Centers for Disease Control and Prevention (CDC) recommends a "Detect and Protect" strategy: detect CRE patients through systematic surveillance and protect patients by preventing transmission of CRE through application of appropriate infection control precautions when such patients enter a health care facility (*14*). Because a patient is often cared for at multiple health care facilities (*15*,*16*), ensuring that information follows a patient is challenging: survivors of prolonged intensive care unit (ICU) treatment go through a median of 4 facility care transitions, including non–acute care facilities, within 1 year (*16*). To improve the effectiveness of the Detect and Protect strategy, information needs to be shared routinely among facilities, but information sharing often is suboptimal (*4*,*17*). Innovative tools to automate information sharing have been developed (*18*) but have focused on hospitals; comprehensive systems are needed that extend beyond acute care hospitals and encompass large geographic regions.

Before 2013, the Illinois Department of Public Health (IDPH) had limited information about the epidemiology of CRE. A CDC-funded surveillance activity (REALM project) (*5*), consisting of point prevalence studies of CRE carriage among ICU patients in Chicago acute care hospitals and all patients in long-term acute care hospitals (LTACHs) was ongoing; however, prevalence data were limited to Chicago and did not include patients outside the ICU or in long-term care facilities (LTCFs). The first function of the XDRO registry was to provide a mechanism for standardized reporting of CRE carrier patients from all health care facilities throughout the state.

In November 2013, IDPH launched a public health informatics tool called the XDRO registry (http://www.xdro.org), designed to facilitate information exchange throughout health care facilities in Illinois. The first function of the registry was to provide a mechanism for standardized reporting of patients in whom CRE was detected. The registry, an electronic platform for CRE information

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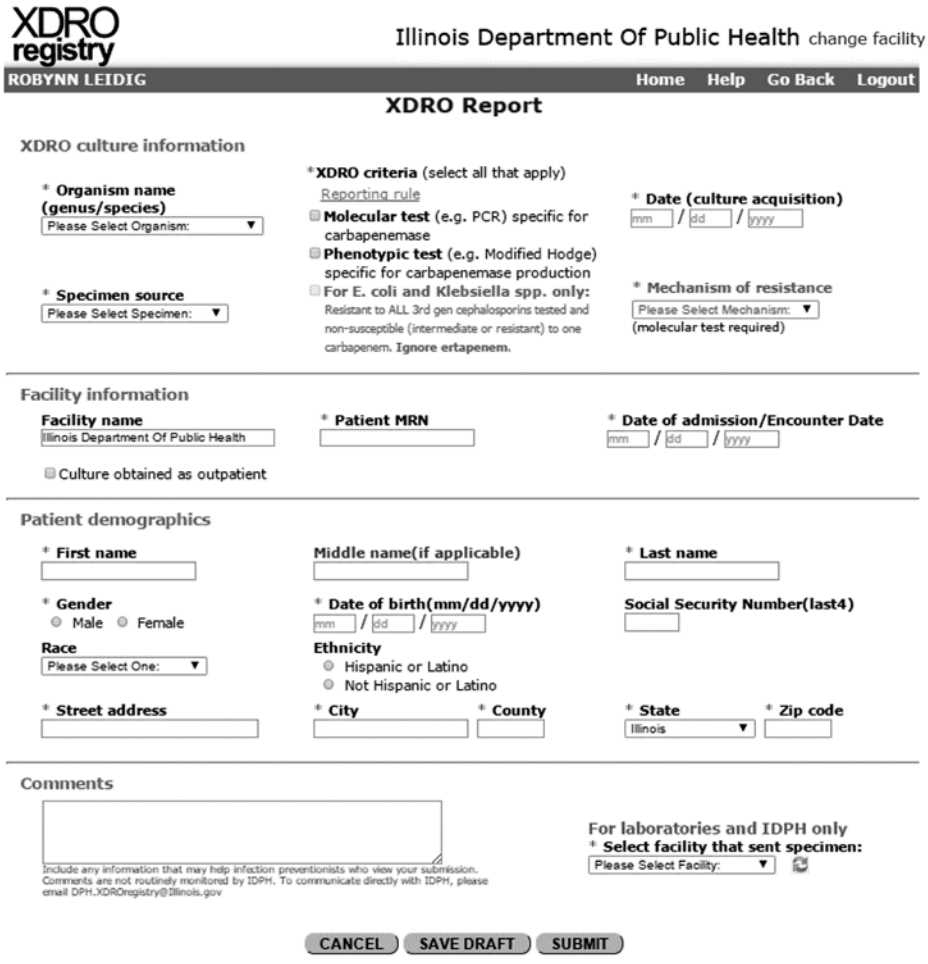


**Figure 1.** Display of Illinois XDRO registry's submission page. Completion of 1 report is contained within this single page. Asterisk indicates required fields. Field names in gray font (i.e., *Escherichia coli* and *Klebsiella* spp. criterion and mechanism of resistance) are conditioned on prior responses, organism name and XDRO criteria, respectively. The field "For laboratories and IDPH only" is not visible for other users; this field enables public health and reference laboratories to input isolates for facilities that have not submitted a report. XDRO, extensively drug-resistant organism.

exchange, receives reports (Figure 1) of CRE in accordance with a state-enacted surveillance rule (Table 1) and centrally stores patient-specific CRE information (Figure 2). The registry was developed through collaboration among public health agencies (federal, state, city, and local), informatics specialists, infection control professionals, microbiologists, and academic researchers. We report our experience designing and implementing the Illinois XDRO registry.

## Registry Development

### Partnership Development and Key Participants

In response to the emergence of CRE in Illinois (*5*), we conceptualized and began developing the XDRO registry in early 2011; the registry went live November 1, 2013. The registry was conceived of and developed by a partnership among public health, academia, infection preventionists (i.e., infection prevention specialists in healthcare facilities), and a nonprofit public health informatics entity. Given its jurisdiction over reporting of communicable diseases, IDPH sponsored the registry. The CDC Chicago Prevention Epicenter provided expertise in designing and implementing the registry. Medical Research Analytics and Informatics Alliance (MRAIA), a 501(c)3 entity designated as an agent of IDPH for public health reporting and related activities, developed and hosted the Web interface, database, and software application for automating alerts. IDPH expanded the Chicago Department of Public Health's CRE advisory group to a statewide task force to gather input from relevant disciplines.

### Design Rationale: CRE Reporting Considerations

#### Mandated versus Voluntary Reporting

One early decision centered on whether the registry should be a voluntary or a mandated reporting system. A voluntary system had the advantage of relatively rapid deployment but would have had incomplete reporting, particularly from facilities not already actively engaged in submitting case reports to IDPH. Instead, motivated by anticipated improvements in reporting adherence, we pursued a mandated

**Table 1.** CRE definition used in the XDRO registry, Illinois, USA*

| |
|---|
| *Enterobacteriaceae* (e.g., *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., *Proteus* spp., *Citrobacter* spp. *Serratia* spp., *Morganella* spp., or *Providentia* spp.) with 1 of the following laboratory results: |
| 1. Molecular test (e.g., PCR) specific for carbapenemase. |
| 2. Phenotypic test (e.g., modified Hodge) specific for carbapenemase production. |
| 3. For *E. coli* and *Klebsiella* spp. only: nonsusceptible (intermediate or resistant) to 1 of the following carbapenems (doripenem, meropenem, or imipenem) AND resistant to ALL of the following third-generation cephalosporins tested (ceftriaxone, cefotaxime, and ceftazidime). Note: ignore ertapenem for this definition. |

*As of November 1, 2013. CRE, carbapenem-resistant *Enterobacteriaceae*; XDRO, extensively drug-resistant organism.

approach that required a change in Illinois' public health rules. Although time consuming, the public vetting process provided transparency and a valuable opportunity for feedback from and acceptance by the infection prevention community. The registry rule was proposed in November 2012 and, after public comment, was finalized in September 2013 with an implementation date of November 1, 2013 (*19*). During the rule-making process, we spent time engaging partners (especially the existing statewide health care–associated infections advisory council), designing and testing the website, and establishing database encryption.

Prior studies in Illinois demonstrated that CRE disproportionately affected chronically ill patients in acute care hospitals (both short- and long-term) and certain LTCFs that cared for mechanically ventilated patients (*5*,*20*). Few patients in the community without health care exposure were colonized with CRE (*20*). On the basis of this evidence, IDPH mandated reporting from all acute care hospitals (short- and long-term) and LTCFs. Laboratories also were required to report CRE, consistent with IDPH's requirement for reporting other infections; this requirement increased reporting of CRE for 2 reasons: 1) laboratories could report for facilities that were not used to or had limited resources for public health reporting, such as LTCFs, and 2) laboratories could report for health care settings not covered by the mandate, such as outpatient clinics. For reference laboratories outside of Illinois, the health care facility must report the isolate.

#### Creating the CRE Definition

We created the Illinois CRE registry definition in accordance with the interim CDC definition proposed in the 2012 CDC CRE Toolkit (*14*). In general, CRE definitions are designed to identify the subset of CRE that produces carbapenemases (rather than noncarbapenemase mechanisms of resistance, such as membrane permeability changes) because carbapenemase-producing *Enterobacteriaceae* have a greater propensity to cause outbreaks. Because the definition does not have perfect specificity for carbapenemase-producing *Enterobacteriaceae*, we retained the term CRE. Although the priority of many surveillance efforts is high sensitivity (i.e., capturing most true cases), we preferred specificity (i.e., few false positives) because the XDRO registry shares information among providers with the intent of implementing infection control precautions, which might negatively affect some patients. Thus, we chose to restrict the antimicrobial drug susceptibility testing criterion (Table 1) to *Escherichia coli* and *Klebsiella* species, because other *Enterobacteriaceae* can have intrinsic imipenem nonsusceptibility (*14*). Other reportable *Enterobacteriaceae*, such as *Enterobacter* species, require a more specific microbiologic test (such as a molecular or phenotypic confirmatory test) (*21*).

We also decided to make the first CRE-positive culture per patient stay reportable to the registry, regardless of whether the patient previously had been reported. We accept multiple reports for a patient to provide accepting facilities with information about when the patient was last reported as a CRE carrier. There is no time limit on patient retention in the registry because duration of CRE carriage is unknown and studies suggest prolonged and possibly indefinite carriage (*22*–*24*).

### Design Rationale: Information Exchange Considerations

Before the registry, the process for sharing patient-specific CRE information among health care providers during direct facility-to-facility patient transfer was by written or verbal communication; such communication was inconsistent. Even with complete communication among facilities during direct facility-to-facility transfer, such communication would affect only the ≈20% of interfacility patient sharing that is direct (*15*). The XDRO registry improves interfacility sharing of CRE information across serial health care facility visits by enabling authorized

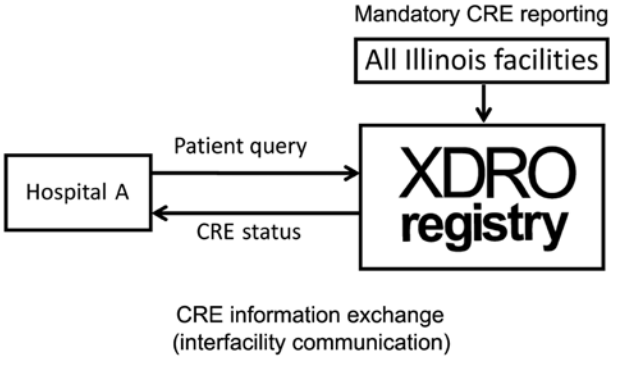


**Figure 2.** Conceptual framework of the XDRO registry, Illinois, USA. XDRO, extensively drug-resistant organism.

personnel at any Illinois health care facility to query whether a patient of unknown CRE status has been reported to the registry (Figure 3).

### Patient Privacy

Sharing patient-level CRE status raised patient privacy concerns. Patients routinely sign consent for their information to be shared for direct facility-to-facility patient transfers, but no explicit authorization is granted for sharing information across disconnected visits (*25*). However, infection control information is unique in that sharing information among health care facilities is expected to occur to prevent spread of antimicrobial drug–resistant organisms to persons at the receiving facility, and electronic alert systems to identify such patients are promoted as a means of protecting patients. After consultation with public health legal counsel at IDPH and CDC, we interpreted the following Health Insurance Portability and Accountability Act provision under General Public Health Activities as authority to build a public health registry and enable interfacility communication to prevent XDRO transmission: "The Privacy Rule permits covered entities to disclose protected health information, without authorization, to public health authorities who are legally authorized to receive such reports for the purpose of preventing or controlling disease, injury, or disability. This would include, for example, the reporting of a disease or injury; reporting vital events, such as births or deaths; and conducting public health surveillance, investigations, or interventions" (*26*). Previous effective interventions support the idea that successful control of XDRO transmission requires active communication among facilities (*8*,*27*).

### Patient Identifier

Exchange of patient information across facilities requires a reliable patient identifier. In Illinois, as in most states, no established master patient identifier existed. We could not rely on Social Security numbers because many patients do not report a number and, for privacy reasons, some hospitals prohibit staff from viewing Social Security numbers. Thus, we combined patient name and date of birth to create an identifier (*28*), which simplified querying patients because such information is routinely accessible to hospital staff.

For automated bidirectional exchange of registry data among health care facilities, we developed software that creates a 1-way hash. We use a deterministic match on the hashed identification (ID); that is, we require exact replication of the name plus date of birth (*28*). We create 3 hashes that all include date of birth but vary on completeness of name, as follows: 1) full first + last names, 2) first initial of first name + full last name, or 3) full last name alone. Regardless of which query matched, we display the patient's first and last name to the user. When a patient match is reported by using a manual query, the registry provides a disclaimer that the infection preventionist needs to verify the patient's CRE status. Verification options include comparing the patient's registry and admission address, asking the patient or family member whether the patient had been in the reporting health care facility at the time of CRE occurrence, or retesting the patient for CRE carriage.

### XDRO Registry Access

Nearly all communicable diseases in Illinois are reported to the Illinois National Electronic Disease Surveillance

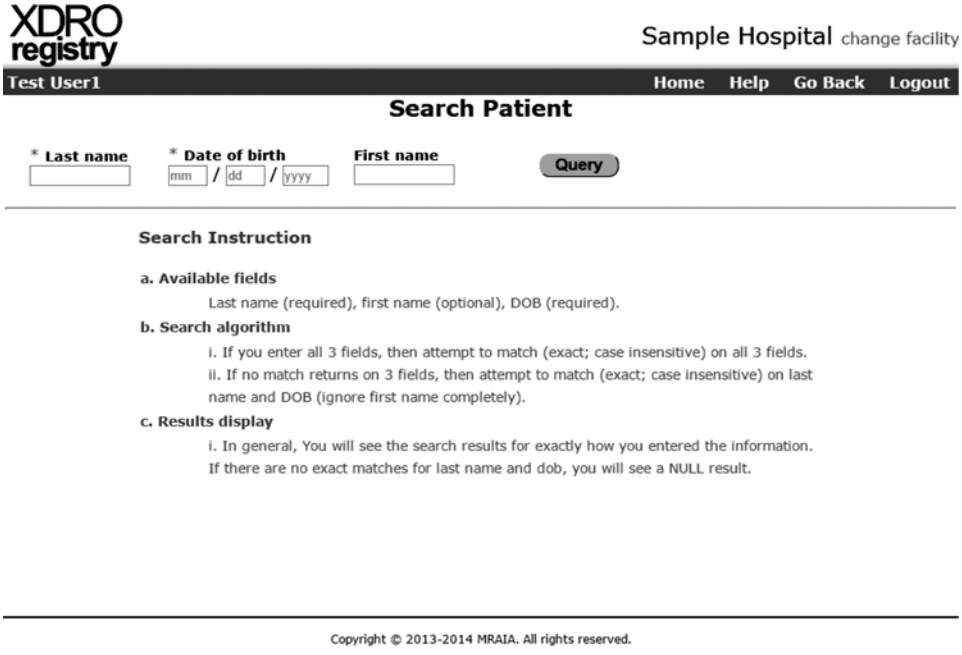


**Figure 3.** Illinois XDRO registry query page. The patient's last name and date of birth are required to execute a search. CRE, carbapenem-resistant *Enterobacteriaceae*; XDRO, extensively drug-resistant organism.

System (I-NEDSS). Access to I-NEDSS is controlled through a password-protected Web portal. We paired the XDRO registry with the I-NEDSS application through the same Web portal, such that authorized I-NEDSS users automatically are given access to the registry. All user permissions are managed through IDPH's existing security infrastructure; the registry stores authentication and activity logs for audit. To protect patient privacy, and because CRE information requires verification by infection control staff, we limit registry access to personnel authorized to electronically submit reportable diseases (i.e., usually infection preventionists at hospitals, designated reporters in free-standing laboratories, and selected persons at LTCFs).

### Technical Infrastructure

The XDRO registry is built on a Web-based platform. The home page (http://www.xdro.org) is publically accessible and displays language for the rule, training materials, frequently asked questions, and a password-protected link to access the registry. The registry is housed on co-located servers (Prominic Inc., Champaign, IL, USA) and managed by personnel at IDPH and the Medical Research Analytics and Informatics Alliance.

## Challenges and Solutions

We have encountered numerous challenges during development of the XDRO registry (Table 2). We highlight several challenges that other public health agencies interested in building a similar registry are likely to encounter.

### Need for a Standardized Unique Health Care Facility Identifier

Accurate identification of facilities is critical for detecting regional clusters of XDROs, assigning reports from reference laboratories to the correct institution, and enabling interfacility communication of alerts; however, no comprehensive unique identification system existed for health care facilities. Although all facilities have a

**Table 2.** Selected challenges encountered and solutions offered during development of a statewide XDRO registry, Illinois, USA*

| Challenge | Solution |
|---|---|
| Legal and regulatory: sharing patient CRE information without explicit informed consent | Public health rule written to authorize reporting/sharing of CRE information, as allowed under HIPAA 45 CFR 164.512(b) |
| Technical, security | |
| Securely maintain username/password permissions | IDPH maintains permissions through existing portal infrastructure in parallel with the I-NEDSS application. User table synchronized with XDRO registry permissions. |
| Electronic laboratory reporting of CRE results | Not implemented; standardized values not defined for all CRE criteria. Custom codes need to be created. Reconciliation between electronic and manual reports will require development. |
| Data accuracy | |
| Susceptibility criterion exclusive to *Klebsiella pneumoniae* and *Escherichia coli*. Selected inappropriately for other organisms | To prevent users from including other species for this "susceptibility criterion," this criterion could not be selected unless *K. pneumoniae* or *E. coli* were chosen as the organism. |
| No master patient identifier available | Combinations of patient last name, first name, and date of birth used as an identifier (Figure 3). Disclaimer to hospital staff to confirm matched patient queries. |
| No universal health care facility identifier available | We use existing IDPH facility codes. LTCFs that do not have I-NEDSS access do not have an identifier and are encouraged to enroll in I-NEDSS. |
| CRE events are entered without systematic validation of data entry | Web entry form has logic embedded to minimize data entry errors. A microbiologic validation of a subset of CRE isolates will be performed in 2015. |
| Single users reporting for multiple facilities | Facility drop-down list created for users who report from multiple facilities. User–facility relationships managed by email request to the registry and human verification. |
| Non-*Enterobacteriaceae* entered through free-text option | The free text option was removed. *Pseudomonas* spp. were the most common non-*Enterobacteriaceae* entered. |
| Work flow | |
| Manual query function is time consuming | Manual querying is most appropriate for facilities with few admissions (e.g., LTCFs). IDPH is developing an automated query system for large facilities. |
| Administratively linked, geographically distinct facilities assigned same code | Request facilities to submit reports as distinct facilities. |
| CRE definition changes | CDC has proposed new criteria for identifying CRE, which requires updating website design and rules. |
| Health departments want to edit cases | Developed after the launch and for now restricted to a few users at the state health department who understand when edits and entries are appropriate. |
| Reference laboratories report CRE events for health care facilities | Each reference laboratory designates a reporter for the registry. Reports linked to individual facilities through a customized drop-down list during submission process. |

*CDC, Centers for Disease Control and Prevention; CRE, carbapenem-resistant *Enterobacteriaceae*; HIPAA, Health Insurance Portability and Accountability Act; IDPH, Illinois Department of Public Health; I-NEDSS, Illinois Notifiable Electronic Surveillance System; LTCF, long-term care facility; XDRO, extensively drug-resistant organism.

National Provider Identifier (NPI) number, that number can be associated with billing units within facilities, so that a single hospital can have multiple associated NPIs. We elected to use an existing set of identifiers known as "site codes" maintained by IDPH to link I-NEDSS users to their institution (IDPH controls access to their secure portal). An advantage of using IDPH site codes was that users with security clearance for reporting to I-NEDSS were automatically granted XDRO registry access, linked to their established I-NEDSS facility.

Although most acute care hospitals had unique site codes, LTCFs historically had reported to I-NEDSS through local health departments without a site code. The absence of LTCF site codes presented a challenge because these facilities often use reference laboratories to detect CRE. Because we could not list unregistered facilities, we had to rely on free text, which complicates cluster detection. We permit reference laboratories to create a list of "favorites" to minimize the variability inherent to free text entries. Also, we actively encourage LTCFs to register in the I-NEDSS system.

Another challenge to using site codes was that sometimes geographically distinct facilities shared a site code. The primary reason for shared site codes was that some health care systems elected to use a single site code across geographically distinct facilities for public health reporting. Because most other reportable diseases are tracked primarily by patient address rather than reporting facility, facility location usually is unnecessary. To correct this problem, IDPH actively reached out to such facilities to encourage assignment of unique site codes.

#### Lack of Routine Validation of CRE Events

A limitation of our system is that, for several reasons, reported CRE isolates are not routinely validated. However, the goal of the registry is to facilitate interfacility communication, and in current practice, patients are deemed CRE-positive by infection preventionists and laboratory personnel on the basis of laboratory criteria. Our long-term vision is to automate reporting with minimal manual entry (i.e., automated electronic laboratory reporting of CRE), which would occur before microbiologic validation of CRE. Given the relatively large number of CRE isolates in Illinois, it was not feasible for a central laboratory to validate every isolate. Instead of routine validation of each isolate, we ask each laboratory to submit a sample of CRE isolates (5 per laboratory) to a reference laboratory to validate organism identification, susceptibility testing, and carbapenemase production.

#### Time-Consuming Manual Queries

The XDRO registry is designed to enable infection preventionists to query the registry to determine whether newly admitted patients carry CRE. In practice, because manually searching the registry for each admitted patient is time consuming, comprehensive manual queries are likely only for facilities with few patient admissions (e.g., LTCFs or LTACHs). Large facilities can query a subset of high-risk patients.

To realize the fundamental goal of rapid notification of infection preventionists, we are piloting automated CRE alerting from the registry. Health care facilities participating in automated alerting electronically transmit an encrypted list of patient admissions that periodically are matched to the registry (e.g., hourly or daily). When a match is found between an admitted patient and the registry, authorized personnel at the admitting health care facility receive an email directing them to log into the registry and view their alert history; the email contains no patient identifiers.

#### Registry Updates and Maintenance

We rapidly address XDRO registry concerns through close collaboration between IDPH, CDC Prevention Epicenter investigators, and the informatics team. During development and the initial year, we scheduled weekly conference calls to discuss user concerns, make website modifications, and plan educational outreach. In the second year, we scheduled meetings every 2 weeks. Users contact IDPH with registry concerns through a dedicated email address or by phone; the contact information is available through the XDRO website. The Illinois CRE task force of local experts in microbiology, infection prevention, and public health provides ongoing counsel. Training webinars were conducted at the time of registry launch, and refresher webinars continue periodically; training materials (including recorded webinar sessions) are accessible through the XDRO registry website.

CDC continues to evaluate the CRE definition as new data and diagnostic tests become available (*29*). As definitions evolve, the registry must accommodate changes. Modifications will require substantial revisions to the website, reeducation of users, and changes in rules for automated alerts. Changing definitions also will make it difficult to follow trends in the number of CRE cases reported. The registry platform enables expansion to other organisms; in particular, the security, search functionality, report distribution to public health, and automated notifications will scale well. However, we will have to develop new reporting rules, web pages, and data tables for additional organisms.

### First Year of Registry

To describe XDRO registry activity, we analyzed the first full year of de-identified data (November 1, 2013–October 31, 2014). For patient and organism descriptive analysis, data were deduplicated at the patient level so that only the first report per patient was retained for analysis.

During the initial 12 months, 1,557 reports were submitted to the XDRO registry, an average of 4.3 per day. These reports contained 1,095 unique patients (1.4 entries for each individual patient). Data were entered from 173 unique facilities: 115 (64%) of 181 registered acute care hospitals, 5 (56%) of 9 LTACHs, 46 LTCFs, and 7 reference laboratories. For some health systems, a single user accessed the registry for several facilities. Because each user's logon is linked to a single primary facility, we have incomplete counting of the number of facilities that have reported to the system.

The median age of reported patients was 64 years (interquartile range 54–75 years; range 11–≥90 years); 52% of patients were female. For ≈43% of unique patients, a mechanism of resistance was known: of those, most common was *K. pneumoniae*–producing carbapenemase (KPC) (461 [98%] of 472), followed by New Delhi metallo-β-lactamase (NDM) (11 [2%] of 472). Most KPC-producing *Enterobacteriaceae* were *Klebsiella* species (84%); most NDM-producing *Enterobacteriaceae* were *E. coli* (85%). Specimens collected were from urine (45%); rectum (19%); wound (12%); sputum (12%); blood (6%); and body fluid, tissue, or other (6%). On average, slightly more than 30 unique facilities queried the registry each month.

We assessed registry use through a paper-based survey of hospitals and LTACHs in the Chicago metro area that participated in a separate public health surveillance project (*6*). During 1 survey period (January–July 2014), hospitals designated an infection preventionist to answer a 15-item written questionnaire about CRE control, of which 3 questions focused on the facility's use of the registry. Twenty-one (88%) of 24 acute care hospitals and all 7 LTACHs responded. Of the respondents, 86% of acute care hospitals and 100% of LTACHs reported having at least 1 person registered to access the registry. Fifty-five percent of hospitals and 43% of LTACHs had queried the status of a CRE-unknown patient. Most acute care hospitals did not routinely query (59%) or queried occasionally (32%); none queried every admitted patient. In contrast, 2 (29%) of 7 LTACHs queried all patients on admission. Ninety-six percent of hospitals expressed interest in automated CRE alerts.

## Future Directions

Future efforts to improve the registry fall primarily under 2 domains: facility alerts and cluster detection for public health. To automate CRE alerting, we are exploring data interfaces between health care facilities and the registry in a way that can scale to many facilities. For example, we are working with a surveillance software vendor common among health care facilities in Illinois. For health care facilities without a vendor solution, we install our hashed ID software system locally so that no protected health information is transmitted to the registry.

IDPH receives weekly automated reports that are manually reviewed to detect clusters or identify reports of uncommon resistance mechanisms. We are exploring enhanced regional situational awareness of potential CRE clusters by using statistical programs (e.g., SaTScan, http://www.satscan.org) that detect spatial or space-time CRE clusters. For example, by geocoding all defined health care facilities and following facility-specific CRE counts across time, individual facilities or groups of facilities can be surveyed for statistically significant changes in CRE reporting. Clusters can be defined on the basis of geographic distance (e.g., all health care facilities within a certain radius) or can be defined within patient sharing networks (e.g., health care facilities that commonly share patients, even if geographically disparate). Such clusters can trigger additional investigation by public health epidemiologists to determine whether an outbreak is occurring.

## Conclusions

CRE surveillance and interfacility communication are recommended for regional infection control but difficult to achieve in practice. We formed a unique partnership among public health, academic investigators, and a nonprofit entity to develop an informatics solution to these challenges. The XDRO registry is an example of a technology-based public health tool that can facilitate CRE detection and communication.

### Acknowledgments

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Dr. Trick is the director of the Collaborative Research Unit for the Cook County Health & Hospitals System. He has an ongoing interest in public health informatics, in particular improving efficiency and reliability by automating historically manual processes.

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# Induction of Multidrug Tolerance in *Plasmodium falciparum* by Extended Artemisinin Pressure

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*Plasmodium falciparum* resistance to artemisinin derivatives in Southeast Asia threatens global malaria control strategies. Whether delayed parasite clearance, which exposes larger parasite numbers to artemisinins for longer times, selects higher-grade resistance remains unexplored. We investigated whether long-lasting artemisinin pressure selects a novel multidrug-tolerance profile. Although 50% inhibitory concentrations for 10 antimalarial drugs tested were unchanged, drug-tolerant parasites showed higher recrudescence rates for endoperoxides, quinolones, and an antifolate, including partner drugs of recommended combination therapies, but remained susceptible to atovaquone. Moreover, the age range of intraerythrocytic stages able to resist artemisinin was extended to older ring forms and trophozoites. Multidrug tolerance results from drug-induced quiescence, which enables parasites to survive exposure to unrelated antimalarial drugs that inhibit a variety of metabolic pathways. This novel resistance pattern should be urgently monitored in the field because this pattern is not detected by current assays and represents a major threat to antimalarial drug policy.

During the past decade, increased commitment and investments in malaria control have markedly reduced malaria-related illness and death in many malaria-endemic areas (*1*). This progress is threatened by emergence of resistance of *Plasmodium falciparum* to artemisinin derivatives used in combination with another drug as first-line therapy for uncomplicated malaria. *P. falciparum* resistance to these derivatives is widespread across Southeast Asia (*1*–*6*) and has been reported in other parts of the world (*7,8*). Artemisinin resistance decreases parasite clearance rates and exposes larger numbers of parasites to antimalarial drugs in vivo, but whether it drives selection of higher-grade artemisinin resistance or resistance to the partner drug is unknown. To address this critical question and anticipate potential changes upon prolonged pressure, models are needed. We used the unique in vitro evolution model of F32-ART parasites selected from the African F32-Tanzania clonal line by using multiple dose-escalating artemisinin pressure to study the effect of extended artemisinin pressure on susceptibility to other antimalarial drugs.

We previously reported that artemisinin resistance results from the capacity of young intraerythrocytic F32-ART parasites (ring-stage parasites) to arrest their cell cycle and enter a quiescence state (*9,10*). This finding was also observed with artemisinin-resistant parasites from Cambodia (*11,12*). Acquisition of a point mutation in the propeller region of the K13 protein after ≈30 drug pressure cycles was on the critical path to artemisinin resistance in the F32-ART lineage (*10*). Genome editing studies confirmed the central role of the K13 locus in artemisinin resistance of the F32 parasites, as well as clinical isolates from Cambodia and diverse laboratory lines (*10,13*). A mutant K13 propeller domain has now been associated with artemisinin resistance in Cambodia and in the Greater Mekong Region (*3*–*6,14,15*). Thus, the F32-ART experimental evolution model proved to be highly relevant in understanding *P. falciparum* artemisinin resistance in the field.

Here, we report the susceptibility profile of F32-ART5 parasites selected after 5 years of escalating artemisinin pressure and assess their susceptibility to this drug and other endoperoxides, as well as unrelated molecules from different chemical classes that inhibit distinct parasite metabolic pathways. We monitored survival and recrudescence after drug exposure, in addition to common monitoring of proliferation. We show that prolonged pressure with only artemisinin results in parasites with a novel pluriresistance phenotype that is highly reminiscent of multidrug tolerance

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of persister bacteria. Dormancy or stress-induced quiescence is a hallmark of bacterial persistence against a variety of antimicrobial drugs (*16*), and periodic exposure to high doses of bactericidal drugs selects increased levels of persister bacteria (*17,18*). An analogous multidrug tolerance/resistance is induced in malaria parasites by extended exposure to high doses of artemisinin, which provides parasites with the capacity to survive lethal doses of diverse classes of antimalarial drugs, including molecules used as drug partners in currently recommended first-line combination therapies.

## Materials and Methods

### Chemicals and Drugs

Chloroquine diphosphate, mefloquine, quinine, and pyrimethamine were obtained from Sigma-Aldrich (Saint-Quentin-Fallavier, France). Artemisinin was obtained from TCI Europe N.V. (Eschborn, Germany) and atovaquone from GlaxoSmithKline (Brentford, UK). Artesunate was obtained from Sanofi (Paris, France) and artemether from Rhone Poulenc Rorer (Paris, France). Dihydroartemisinin and monodesetylamodiaquine (amodiaquine/AQ) were obtained from WWARN (http://www.wwarn.org/). Artemisone was synthesized according to published protocols (*19*).

### Parasites and Parasite Culture

For selection of artemisinin-resistant *P. falciparum* parasites, asynchronous cultures of the F32-Tanzania clone were adjusted to a parasitemia of 5%–7% and grown in the presence of increasing doses of artemisinin (range 10 nmol/L–9 μmol/L) for 24 h for the first 3 years of drug pressure, which resulted in F32-ART3 (*9*). In the next 2 years, each drug-pressure cycle lasted 48 h (drug dose range 9 μmol/L–18μmol/L), which resulted in F32-ART5 (*10*) (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/10/15-0682-Techapp1.pdf). To ensure maintenance of the phenotypic characteristics, parasites were cultivated under regular drug pressure. F32-ART5 parasites studied were collected during the period extending from the 120th to the 123rd artemisinin pressure cycle. Phenotypic and genomic analysis showed no differences in parasites from these 4 (120–123) drug pressure cycles. F32-ART5 and its sibling drug-sensitive F32-TEM, cultured without artemisinin, were cultivated in parallel by using the method of Trager and Jensen with modifications (*9,20*).

### Standard Isotopic Drug Susceptibility Assay

The standard isotopic 48-h $^{3}$H-hypoxanthine-based test (*21*) was used, with minor modifications, to assess the sensitivity of F32-ART5 and F32-TEM lines to 10 antimalarial drugs (*9,20*). The 50% inhibitory concentrations ($IC_{50}$s) were determined after 48 h of incubation by using ICEstimator software (http://www.antimalarial-icestimator.net).

### Drug Recrudescence Assay

Synchronized F32-TEM and F32-ART5 ring stages were exposed to drug for 48 h, washed, and placed in drug-free medium. A preliminary drug screening (range 2-fold to >4,000-fold the $IC_{50}$) enabled determination of the most appropriate antimalarial drug dose to discriminate the phenotype response of both lines in the recrudescence assay. Parasitemia was monitored daily to determine the time to recrudesce to the initial parasitemia (*9*). To evaluate artemisinin resistance of older parasite stages, 24-hour-old trophozoites were exposed for 48 h to 3.5 μmol/L artemisinin, and recrudescence was monitored.

### Ring-Stage Survival Assay

For the ring-stage survival assay (RSA), ring-stage parasites (0–3 h postinvasion [$RSA^{0–3\ h}$] or 13–16 h postinvasion [$RSA^{13–16\ h}$]) from highly synchronous cultures were exposed to 700 nmol/L dihydroartemisinin or 0.1% dimethyl sulfoxide (DMSO) for 6 h, washed, and cultivated for 66 h in standard culture conditions as described (*10–12*). Survival rates were calculated after microscopic examination of Giemsa-stained blood smears as the proportion of viable second-generation parasites in wells containing drug compared with that in wells containing DMSO (*11,12*). Blinded slides were read by ≥2 expert microscopists. $RSA^{0–3\ h}$ was also performed with 0–3-h postinvasion ring-stage parasites exposed for 6 h to atovaquone (3 μmol/L) or amodiaquine (0.3 μmol/L).

### Ring-Stage Growth Arrest Assay

The ring-stage growth arrest assay (*11*) was used with modifications. In brief, synchronized ring-stage cultures (3%–5% parasitemia, 2.5% hematocrit) were treated with 5% sorbitol to lyse mature stages immediately after a 24-h exposure to 11 μmol/L artemisinin or 0.1% DMSO. Cultures were then resuspended in drug-free culture medium, and parasite counts were monitored microscopically daily until day 28 for sorbitol-treated or non–sorbitol-treated cultures exposed to artemisinins. Time to recrudescence was the time to recrudesce to the initial parasitemia. For the control (DMSO) culture, results were expressed as percentage parasite density 24 h after treatment with sorbitol compared with that for cultures not treated with sorbitol. Results were determined in 5 independent experiments.

### Whole-Genome Sequencing

Whole-genome sequencing of F32-ART5 collected after 123 pressure cycles was performed by using paired-reads sequencing technology (Illumina, Inc., San Diego, CA, USA). Sequences were compared with those of F32-ART5 collected after 120 pressure cycles (*10*), F32-TEM, and reference strain 3D7, as reported (*10*).

**Table 1.** Susceptibility of *Plasmodium falciparum* F32-ART5 and F32-TEM lineages to 10 antimalarial drugs*

| Drug | F32-TEM | | | F32-ART5 | | | p value† |
|---|---|---|---|---|---|---|---|
| | $IC_{50}$ | $IC_{90}$ | $IC_{99}$ | $IC_{50}$ | $IC_{90}$ | $IC_{99}$ | |
| Artemisinin | 14.2 (13–19.5) | 20.9 (16.3–26.4) | 29.2 (20.7–39.2) | 17.7 (14.2–21.9) | 31.2 (23–37.2) | 50.7 (37.2–63.3) | 0.686 |
| Artesunate | 3.9 (2.7–5.1) | 6.4 (4.7–7.9) | 11.1 (9.4–13.4) | 3.8 (3.2–5.6) | 6.1 (4.7–7.4) | 11.1 (7.7–13.4) | 0.886 |
| Artemisone | 0.6 (0.3–0.9) | 0.7 (0.5–0.9) | 0.9 (0.6–1.1) | 0.5 (0.3–0.6) | 1.2 (1.1–1.3) | 3.5 (3.3–3.8) | 1.000 |
| Artemether | 7.2 (7–7.5) | 9 (8.9–9.2) | 11.5 (11.2–11.7) | 7.2 (6.8–7.5) | 8.9 (8.7–9.2) | 11.4 (11.1–11.7) | 1.000 |
| Chloroquine | 32.9 (23.64–45.6) | 55.9 (42.9–58.7) | 66.2 (52.1–72.1) | 28.2 (24.9–33.5) | 46.2 (44.7–52.4) | 83.4 (76.8–100.5) | 0.886 |
| Quinine | 105.8 (77.8–136.1) | 302.7 (219.9–420.7) | 938 (718.5–1,482.5) | 118.8 (84.2–156.5) | 341.6 (259.7–421.5) | 1,026 (742.2–1,439) | 0.486 |
| Amodiaquine | 25.1 (17.1–33.1) | 53.1 (51.7–54.4) | 113.4 (88.7–138) | 29.6 (28.4–30.9) | 51.8 (47.5–56.2) | 78.05 (66.5–89.7) | 1.000 |
| Mefloquine | 68.2 (54.7–88.2) | 142.3 (130.9–159.6) | 312.9 (224–437) | 63.1 (59.4–73.1) | 114.1 (101–153.7) | 220.9 (161.6–448.5) | 0.886 |
| Pyrimethamine | 105.0 (90–120.1) | 184.6 (167.1–202.1) | 385.9 (288–483.8) | 74.7 (73.3–76.1) | 270.5 (268.7–272.3) | 1,102.6 (1,076–1,129) | 0.333 |
| Atovaquone | 2.8 (2–2.9) | 12.7 (10.8–15.8) | 95.8 (87.2–104) | 1.5 (0.4–3) | 7.5 (2.6–12.9) | 52.1 (31–64.6) | 0.486 |

*$IC_{50}$, 50% inhibitory concentration; $IC_{90}$, 90% inhibitory concentration; $IC_{99}$, 99% inhibitory concentration. Values were obtained by using the standard isotopic susceptibility assay. Drug concentrations are in nanomoles/liter. Values are medians (25%–75% interquartile ranges). All assays were performed in triplicate.
†Data distribution was non-Gaussian. The p values were calculated by using nonparametric Mann-Whitney rank-sum test results for $IC_{50}$s. Similar tests were performed to obtain $IC_{90}$s and $IC_{99}$s, and no differences were observed between F32-ART5 and F32-TEM for any drug tested.

### Statistical Analysis

Statistical tests were performed by using SigmaStat version 2.03 (Heame Scientific Software, Chicago, IL, USA). Recrudescence curves (online Technical Appendix Figure 2) were analyzed by using the Mantel-Cox test and GraphPad Prism software (GraphPad Inc., San Diego, CA, USA) (*5*). Differences in comparisons were considered significant if p values were ≤0.05.

## Results

### Effect of Long-term Artemisinin Pressure on $IC_{50}$s for Antimalarial Drugs

F32-ART5 and F32-TEM (cultivated for the same duration in the absence of drug pressure) parasites had similar, low $IC_{50}$s for 4 artemisinin derivatives in the standard isotopic drug susceptibility assay. The same result was observed for quinolines, an antifolate, or atovaquone (Table 1). Moreover, both lines had similar $IC_{90}$s and $IC_{99}$s for all antimalarial drugs tested. Thus, the standard chemosensitivity assay shows absence of proliferation of F32-ART5 and F32-TEM parasites in presence of all 10 antimalarial drugs tested.

### Survival of F32-ART5 against High-Dose Artemisinin after Drug–Induced Quiescence

In the $RSA^{0–3h}$, in which young ring stages were exposed to 700 nmol/L dihydroartemisinin for 6 h, the survival rate of F32-ART5 parasites was similar to that for F32-ART3, and much higher than survival rates for F32-TEM and F32-ART parasites before acquisition of the K13 M476I mutation (*10*) (Table 2; online Technical Appendix Figure 1). Moreover, survival rates of F32-ART5 parasites after 122 and 123 drug pressure cycles were similar.

In the recrudescence assay, in which parasites were exposed to 11 μmol/L artemisinin or 18 μmol/L artemisinin for 48 h, F32-ART5 recrudesced 11 days earlier than F32-TEM (Figure 1, panel A; Figure 2, panel A; Table 3). This finding is consistent with the previously reported recrudescence profile of F32-ART3 parasites (*9*).

To demonstrate that parasite survival against exposure to artemisinin resulted from quiescence involving a proliferation blockade (*11*), sorbitol treatment was performed immediately after a 24-h exposure of synchronized ring-stage cultures to 11 μmol/L artemisinin in the ring-stage growth arrest assay (*11*). Sorbitol selectively lyses erythrocytes infected with trophozoites and mature parasites, but not young stages. Thus, quiescent forms that do not or minimally develop during the 24-h drug exposure are resistant to sorbitol treatment (*9,11*). The ring-stage growth arrest assay showed that F32-ART5 parasites exposed to artemisinin recrudesced at the same time whether treated with sorbitol (7.5 d, range 6.5–9 d) or not treated with sorbitol (7.5 d, range 6.5–9.5 d). In contrast, sorbitol induced a severe survival loss in the control culture exposed to DMSO, in which parasite maturation had proceeded unimpaired, such that the mean survival of sorbitol-treated parasites was only 16% (range 6%–33%) of the survival observed in DMSO-control cultures not treated with sorbitol.

**Table 2.** RSA values for *Plasmodium falciparum* F32-TEM and F32-ART lineages and recrudescence times for trophozoite parasite stages after a 48-h exposure to artemisinin*

| Artemisinin pressure cycle | Dose, μmol/L | RSA$^{0–3\ h}$ Median survival rate† (IQR) | RSA$^{0–3\ h}$ No. assays‡ | RSA$^{13–16\ h}$ Survival rate† | RSA$^{13–16\ h}$ No. assays‡ | Recrudescence time for trophozoite stage, d Median (IQR) | Recrudescence time for trophozoite stage, d No. assays‡ |
|---|---|---|---|---|---|---|---|
| 0 (F32-TEM) | 0 | 0 (0–0.03)§ | 5 | 0 | 1 | 17.5 (17–18) | 2 |
| 12 | 0.02 | 0 (0–0) | 2 | 0 | 1 | ND | NA |
| 17 | 0.04 | 0 (0–0.07)¶ | 3 | 0 | 1 | ND | NA |
| 48 | 2.7 | 11.7 (10.3–14.6)¶ | 3 | 2.5 | 1 | ND | NA |
| 115 | 8.9 | 6.8 (5.9–15.9) | 3 | 2.1 | 1 | ND | NA |
| 122 | 9 | 12.8 (10.6–14.5)¶ | 3 | 3.8 | 1 | ND | NA |
| 123 | 10 | 9.5 (8.1–11.8)§ | 4 | 2.9 | 1 | 11 (10.3–12.5) | 3 |

*RSA, ring-stage survival assay; IQR, interquartile range; ND, not determined; NA, not applicable.
†Survival rates are expressed as percentage of parasites remaining alive after drug treatment compared with mock-treated culture.
‡Assays that fulfilled criteria for successful culture.
§Significant survival rate difference (by Mann Whitney rank sum test, $p<0.05$) between F32-ART5 and its sibling line F32-TEM.
¶Data were obtained from Ariey et al. (*10*).

### Resistance of Young Ring-Forms and Older Stages of F32-ART Parasites to Artemisinin

We investigated by using RSA the stage-dependent survival capacity of young rings (0–3 h postinvasion) and older rings (13–16 h postinvasion) of the F32-ART lineage at different steps of the selection process (Table 2). We found a parallel increase in survival rates for both stages, although lower rates were observed for older stages. The marked shift of increased survival of young ring-stage parasites previously reported as occurring at approximately pressure cycle 48 coincided with increased survival of older ring-stage parasites, and both rates remained essentially unchanged subsequently.

Drug recrudescence assays showed that F32-ART5 trophozoites recovered more efficiently after a 48 h exposure to artemisinin than F32-TEM trophozoites (11 and 17.5 days, respectively) (Table 2). These data outlined an unsuspected extended age range of stages surviving treatment with artemisinin.

### Selection of Multidrug Tolerance by Long-term Artemisinin Pressure

We reported previously that F32-ART3 showed cross-resistance to artesunate and remained susceptible to chloroquine (i.e., displayed parental-type recrudescence rates after a 48-h exposure to chloroquine in the recrudescence assay) (*9*). Recrudescence assays were performed with F32-ART5 for 4 endoperoxides and a panel of unrelated antimalarial drugs. F32-ART5 showed increased survival rates to high doses of artesunate, artemisone, or artemether, and recrudescence occurred 7.5–14.3 days earlier than for F32-TEM depending on the endoperoxide and

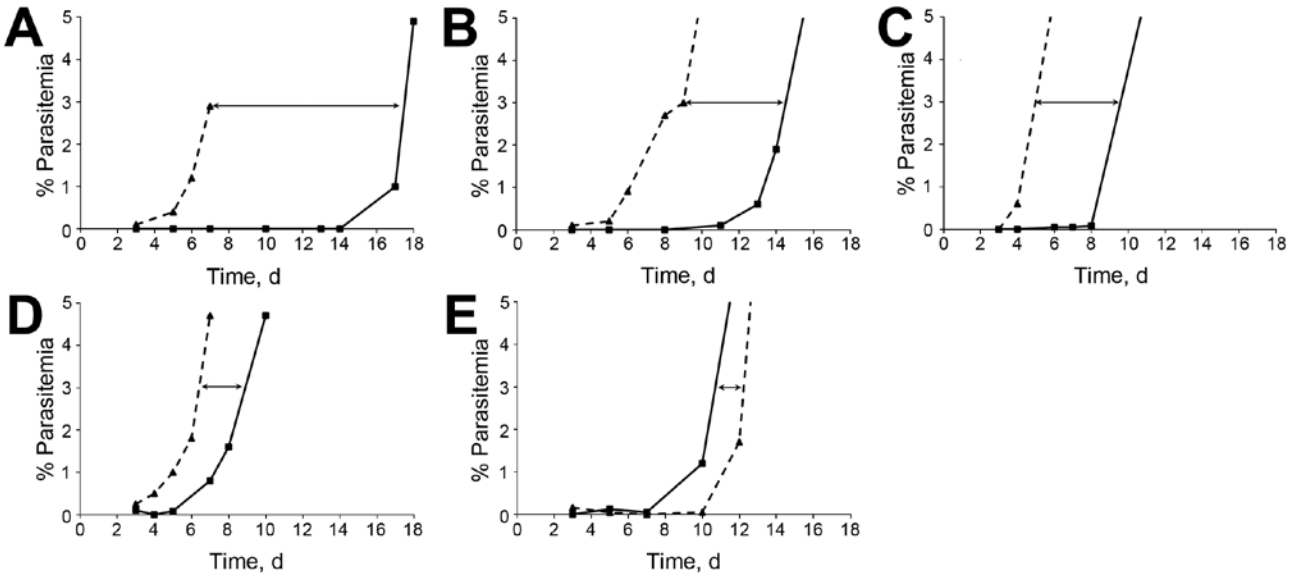


**Figure 1.** In vitro drug survival assays for *Plasmodium falciparum*. Representative curves for kinetic recrudescence of synchronous ring-stage parasites from F32-ART5 lineage (dashed lines) and F32-TEM lineage (solid lines) parasite cultures after a 48-h exposure to A) 11 μmol/L artemisinin; B) 62 nmol/L amodiaquine; C) 241 nmol/L mefloquine; D) 4 μmol/L pyrimethamine; and E) 7 μmol/L atovaquone. Differences in recrudescence between both parasite lines are indicated by doubled-headed arrows.

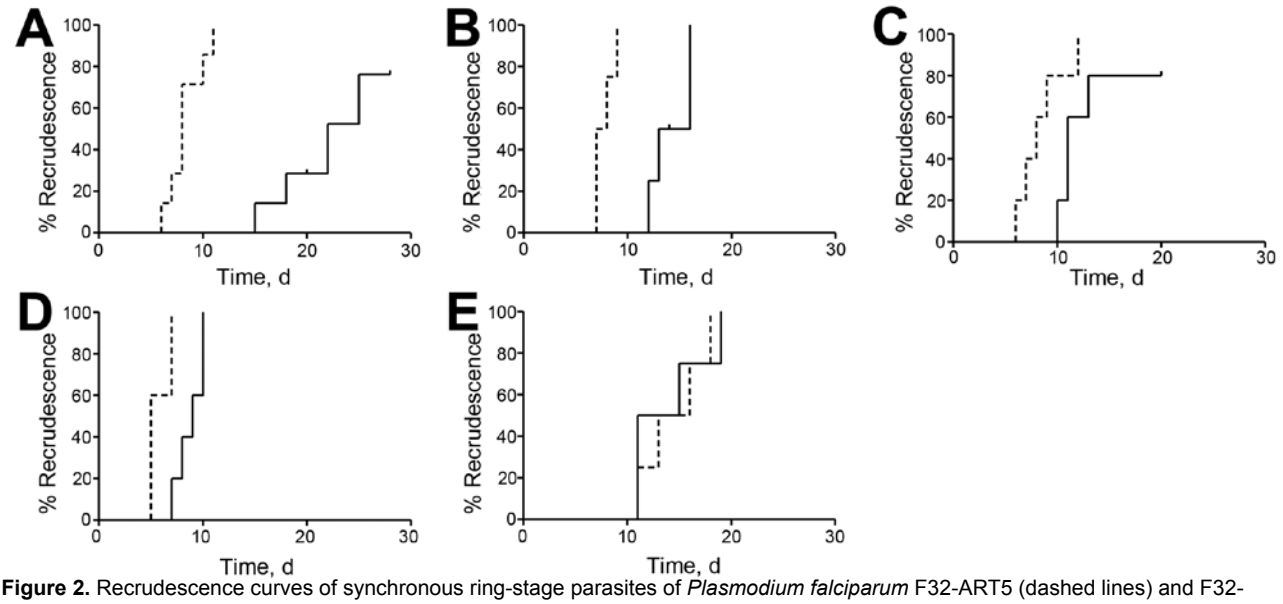


**Figure 2.** Recrudescence curves of synchronous ring-stage parasites of *Plasmodium falciparum* F32-ART5 (dashed lines) and F32-TEM (solid lines) after a 48-h exposure to A) 11 μmol/L artemisinin; B) 62 nmol/L amodiaquine; C) 241 nmol/L mefloquine; D) 4 μmol/L pyrimethamine; and E) 7 μmol/L atovaquone. Curves show the percentage of parasite recrudescence (i.e., cultures having reached day 0 parasite density) vs. time. A log-rank (Mantel-Cox) test was used for statistical analysis, and corresponding p values are reported in Table 3. Small black vertical tick marks indicate individual F32-TEM lines whose recrudescence times have been right-censored because the parasite line did not recrudesce during the monitoring study.

concentration (Table 3; online Technical Appendix Figure 2). The >7 days earlier recrudescence for F32-ART5 exposed to artemether compared with that for F32-TEM was not significant because of small numbers of paired-sample experiments.

Shorter recrudescence time for F32-ART5 were consistently observed after a 48-h pulse of amodiaquine, mefloquine (Table 3, Figure 1,2, panels B, C, respectively) and chloroquine (Table 3; online Technical Appendix Figure 2). For quinine, we found a trend for increased survival of F32-ART5 compared with that for F32-TEM. Improved survival of F32-ART5 after exposure to quinine was further suggested by 5/5 F32-ART5 cultures having recrudesced compared with 3/5 F32-TEM cultures (online Technical Appendix Figure 2). A shorter recrudescence time for F32-ART5 compared with that for F32-TEM was also observed after a 48-h pulse with pyrimethamine (Table 3; Figure 1, panel D; Figure 2, panel D). Therefore, in vitro drug pressure only with artemisinin selects for enhanced survival rates to endoperoxides, quinolines, and an antifolate.

Activity of atovaquone against F32-ART5 parasites was preserved. After a 48-h atovaquone pulse, recrudescence rates for F32-TEM and F32-ART5 were not different (Figure 1, panel E; Figure 2, panel E; Table 3; online Technical Appendix Figure 2). Likewise, RSA performed with atovaquone showed no difference between F32-ART5 and F32-TEM (online Technical Appendix). The same result was observed for RSA performed with amodiaquine (online Technical Appendix).

### Association of Drug Resistance Genes with Multidrug Tolerance

Because phenotypes described were obtained for F32-ART5 parasites collected during the period corresponding to 120–123 pressures cycles, whole-genome sequencing was conducted, and we compared the sequence of F32-$ART5_{123}$ with that of F32-$ART5_{120}$ (*10*). There was no evidence of genotype modification during the 120th–123rd artemisinin pressure cycles (online Technical Appendix Figure 1), which is consistent with highly reproducible phenotypes of various F32-ART5 cultures collected during that period. F32-ART5 did not differ from F32-TEM with regard to resistance gene markers, some of which were wild-type (chloroquine resistance transporter, dihydrofolate reductase, and cytochrome B) and others harbored mutations (multidrug resistance 1, ATPase6, $Na^+/H^+$ exchanger 1, dihydropteroate synthase, multidrug resistance 1 [*mdr1*] and *mdr2*) (online Technical Appendix Table). There was no evidence for *mdr1* gene amplification in F32-ART5 (and its lineage) and F32-TEM.

## Discussion

F32-ART5 parasites have a novel drug resistance profile that is reminiscent of multidrug tolerance profile of bacterial persister cells (*16,22*). Similar to the unchanged

**Table 3.** Recrudescence parameters of *Plasmodium falciparum* F32-ART5 and F32-TEM lineages exposed to 10 antimalarial drugs*

| Drug | Drug dose | No. experiments† | Median (range) recrudescence time, d‡ F32-ART5 | F32-TEM§ | Mean ± SEM difference of recrudescence time, d¶ | p value‡ |
|---|---|---|---|---|---|---|
| Artemisinin | 11 μmol/L | 7 | 8 (6–11) | 22 (15–>28) | 11.5 ± 1.5 | <0.001 |
| | 18 μmol/L | 3 | 7 (6–9) | 19 (17–20) | 11.3 ± 1.3 | 0.024 |
| Artesunate | 1.3 μmol/L | 3 | 8 (7–10) | 16 (15–18) | 8 ± 1.5 | 0.024 |
| | 2.6 μmol/L | 3 | 8 (7–10) | 16 (15–18) | 8 ± 1.5 | 0.024 |
| Artemisone | 1.2 μmol/L | 3 | 8 (7–10) | 20 (18–20) | 11 ± 1.5 | 0.024 |
| | 2.5 μmol/L | 3 | 8 (7–11) | 21 (20–28) | 14.3 ± 1.4 | 0.025 |
| Artemether | 1.7 μmol/L | 2 | 7.5 (7–8) | 15 (15–15) | 7.5 ± 0.5 | 0.089 |
| | 3.4 μmol/L | 2 | 7.5 (7–8) | 16 (16–16) | 8.5 ± 0.5 | 0.089 |
| Chloroquine | 78 nmol/L | 4 | 10 (7–11) | 13 (11–20) | 4.8 ± 1.5 | 0.028 |
| Quinine | 43 μmol/L | 5 | 10 (8–14) | 13 (10–>20) | 2.7 ± 0.9 | 0.086 |
| Amodiaquine | 62 nmol/L | 4 | 7.5 (7–9) | 14.5 (12–16) | 6 ± 0.6 | 0.006 |
| Mefloquine | 241 nmol/L | 5 | 8 (6–12) | 11 (10–>20) | 3 ± 1.1 | 0.044 |
| Pyrimethamine | 4 μmol/L | 5 | 5 (5–7) | 9 (7–10) | 3 ± 0.5 | 0.008 |
| Atovaquone | 3 μmol/L | 5 | 13 (3–27) | 12 (3–21) | −1.4 ± 1.3 | 0.730 |
| | 7 μmol/L | 4 | 14.5 (11–18) | 13 (11–19) | −0.5 ± 0.6 | 0.848 |

*Recrudescence capacity of F32-TEM and F32-ART5, synchronized at ring-form cultures (0–12-h-old parasites), was evaluated after 48 h of drug treatment. After cultures were washed, parasitemia was monitored daily to determine the recrudescence time, defined as the time to reach day 0 parasitemia.
†Each experiment was performed for F32-ART and F32-TEM cultivated in parallel in the same conditions (adjusted to the same initial parasitemia and cultivated with the same lot of erythrocytes and same batch of human serum) to generate paired results. The same number of experiments was performed for each parasite lineage and statistically analyzed.
‡A log-rank (Mantel-Cox) test was used for statistical analysis of recrudescence time in days.
§If no parasites were observed at the end of the experiment, the culture was classified as showing no recrudescence, and the recrudescence day was noted as >d. Parasite counts were monitored microscopically daily until day 28 except when the batch of blood needed to be changed (in this instance, the experiment was stopped earlier).
¶Differences in recrudescence time as Gaussian data.

inhibitory antimicrobial drug concentrations for bacterial persisters compared with those for their clonal parent strains, $IC_{50}$s, $IC_{90}$s, and $IC_{99}$s of F32-ART5 parasites were similar to those of F32-TEM for 10 antimalarial drugs. Thus, these malaria parasites would be classified as drug susceptible to all 10 drugs in standard proliferation assays. However, high survival rates were observed after exposure to 4 endoperoxides, as well as increased survival rates to lethal doses of pyrimethamine and quinolines (amodiaquine, mefloquine, chloroquine, and quinine). Thus, the in vitro experimental evolution model we used indicates that *P. falciparum* can acquire after sustained artemisinin pressure the capacity to survive exposure to diverse antimalarial drugs. This novel resistance profile differs from classical multidrug resistance because parasites do not multiply in the presence of the drug and do not harbor any mutations conferring resistance to pyrimethamine or quinolines. This profile is not a fully generalized tolerance phenomenon because F32-ART5 is still susceptible to atovaquone.

The capacity of parasites to enter quiescence after exposure to artemisinin and readily resume growth after drug removal is central to resistance to artemisinin (*9,11,12*) and is similar to persistence of bacteria after exposure to antimicrobial drugs (*17*). Quiescence, also known as dormancy (for definitions, see online Technical Appendix), reflects the cell cycle–arrested status and decreased parasite metabolism of parasites exposed to artemisinin (*23*). Resistance to artemisinin is also associated with increased constitutive expression of unfolded protein response pathways, which are believed to mitigate toxicity of artemisinin (*24*). Quiescence of ring-stage F32-ART5 parasites, which did not develop during a 24-h exposure to artemisinin, was shown by the insensitivity of these parasites to treatment with sorbitol and unaltered recrudescence time in the ring-stage growth arrest assay. Because recrudescence rates for other endoperoxides tested were similar to those observed for artemisinin (Table 3), we conclude that F32-ART5 survives the toxicity of endoperoxides by similar cellular mechanisms.

During this study, we determined that not only young rings but also older parasites developmental stages of the F32-ART lineage survived exposure to artemisinin, which denoted an extended range of stages able to enter quiescence and withstand artemisinin toxicity. Relaxed ability of F32-ART5 for cell cycle arrest in response to drug-induced cellular stresses might affect increased recrudescence rates after exposure to quinolines and pyrimethamine, which inhibit metabolically active mature stages and yet are highly active in the standard susceptibility assay that monitors parasite multiplication. Consistent with this idea, mefloquine was shown to induce cell cycle delay (*25*), including that for ring-stage parasites (*26*). Moreover, the reduced metabolic activity of quiescent older forms is predicted to decrease toxicity of antimalarial drugs that inhibit parasite metabolic pathways, such as hemozoin formation (inhibited by quinolines) or tetrahydrofolic acid synthesis (inhibited by pyrimethamine). In contrast, atovaquone, which inhibits parasitic mitochondrial electron transfer and consequently reduces mitochondrial electron membrane potential, remained fully active for F32-ART5 parasites. This finding is consistent with results that showed that

parasites retain their basal mitochondrial metabolism during artemisinin-induced quiescence (*23,27*), and that addition of atovaquone to ring-stage parasites does not delay parasite maturation (*26*). Unfortunately, atovaquone resistance is readily selected in the field, being detected a few months after deployment in areas of resistance to artemisinin-based combination therapies (ACTs) (*2*). Nevertheless, our data indicate that drug combinations that include atovaquone could be a useful option in decreasing resistance to ACTs in the field.

Despite its extended age range of stages able to enter quiescence, F32-ART3 was fully susceptible to chloroquine in recrudescence assays conducted with the same protocol (*9*). This finding indicates that tolerance to chloroquine was acquired at a later stage of selection (after the 110th pressure cycle). Whether this tolerance was associated with multidrug tolerance or whether acquisition of multidrug tolerance is a multistep process remains to be clarified. To define more precisely the mechanisms, the number of steps, and putative loci involved in multidrug tolerance, an analysis of the cryopreserved intermediate time points of the F32-ART lineage would be needed. None of the mutations that occurred after fixation of M476I in the K13 locus (online Technical Appendix Figure 1) are analogous with mutations associated with persistence in bacteria or fungi (*16*), and some mutations affect genes of unknown function.

The nonsense mutation in PF3D7_1115700, which encodes falcipain 2a, a cysteine protease involved in hydrolysis of hemoglobin (*28*), is predicted to decrease artemisinin sensitivity of trophozoite stages by impairing artemisinin-induced exacerbation of oxidative stress caused by hemoglobin degradation products (*29*). Absence of this enzyme should also decrease the amount of hemoglobin degradation products essential to activity of quinolines (*30*) and endoperoxides (*19,31*). The nonsense falcipain 2a mutation, which is present in F32-ART3, might contribute to reducing sensitivity of F32-ART5 to artemisinins and some quinolines (other than chloroquine), but it is unlikely for pyrimethamine, whose activity is not related to hemoglobin metabolism.

Reduced sensitivity for chloroquine or pyrimethamine, whose target genes (chloroquine resistance transporter gene and dihydrofolate reductase gene, respectively) are wild type in the F32-ART lineage (online Technical Appendix Table 1), might result from mutations in other loci affected at late stages of artemisinin pressure or reflect decreased toxicity because of reduced metabolic activity of quiescent parasites. Detailed analysis of the small number of genes mutated in F32-ART5 parasites is needed to gain insights about the cellular alterations underlying multidrug tolerance. In addition, investigation of the field polymorphism of these candidate genes might provide indications about possible ongoing selection processes. It is worth noting that none of these genes was as reported as having signatures of recent selection in Southeast Asia (*6*) or Africa (*10,32*). These findings do not preclude that such selection might eventually occur in K13 mutant parasites, including isolates harboring the M476I K13 mutation (*5,33*).

The F32-ART model was relevant for understanding cellular and molecular mechanisms of artemisinin resistance in the field (*9,10*). The finding that not only young ring forms but also older developmental stages survived exposure to artemisinin differs from observations with artemisinin-resistant field isolates from Cambodia, whose increased survival seems restricted to younger stages, although a trend for increased survival of older ring stages was observed (*12*). Whether this finding reflects different selection processes in the field in Cambodia and during the in vitro model used here is unclear. This finding could also reflect intrinsic differences of genetic backgrounds in which resistance mutations emerge because F32-ART has a genetic background from Africa (*10*).

Genome editing studies have shown that K13 mutations and parasite genetic background influence $RSA^{0–3\ h}$ survival rate of young ring stages (*13*). However, survival of older stages has not been studied. Likewise, clearance half-life and $RSA^{0–3\ h}$ survival rates of isolates from Cambodia depend on the K13 mutation type (*10,14*). Extended monitoring of phenotypes of mutant parasites from Southeast Asia to older developmental stages and characterizing survival and recrudescence phenotype of parasites from Africa harboring a mutant K13 locus (*34*) are urgently needed to provide information on possible selection processes for multidrug tolerance in the field. It is also necessary to further characterize the stage-dependent phenotype of rare field isolates harboring the M476I mutation, including analysis of possible multidrug tolerance phenotype. The recent emergence of resistance to piperaquine, an ACT partner drug, in western Cambodia is particularly worrisome in this context (*35*). However, none of the assays currently used to monitor drug susceptibility of field isolates is able to detect this novel multidrug tolerance phenotype.

The pleiotropic effect of artemisinin pressure reported is a major concern for ACT-based drug policy because sustained pressure on artemisinin-resistant parasites may drive selection of artemisinin resistance in older parasite stages and result in decreased efficacy of the partner drug in the field. Apart from the retained efficacy of atovaquone that suggests possible alternative combination treatments, multidrug tolerance of F32-ART5 to mefloquine, amodiaquine, and pyrimethamine in vitro is a serious concern because of large-scale use of these drugs as partner drugs in ACTs. Specific assays should be urgently implemented to monitor this novel phenotype in the field that otherwise will remain undetected by current in vitro assays or molecular markers.

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# Evolutionary and Ecological Characterization of Mayaro Virus Strains Isolated during an Outbreak, Venezuela, 2010

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In 2010, an outbreak of febrile illness with arthralgic manifestations was detected at La Estación village, Portuguesa State, Venezuela. The etiologic agent was determined to be Mayaro virus (MAYV), a reemerging South American alphavirus. A total of 77 cases was reported and 19 were confirmed as seropositive. MAYV was isolated from acute-phase serum samples from 6 symptomatic patients. We sequenced 27 complete genomes representing the full spectrum of MAYV genetic diversity, which facilitated detection of a new genotype, designated N. Phylogenetic analysis of genomic sequences indicated that etiologic strains from Venezuela belong to genotype D. Results indicate that MAYV is highly conserved genetically, showing ≈17% nucleotide divergence across all 3 genotypes and 4% among genotype D strains in the most variable genes. Coalescent analyses suggested genotypes D and L diverged ≈150 years ago and genotype diverged N ≈250 years ago. This virus commonly infects persons residing near enzootic transmission foci because of anthropogenic incursions.

The family *Togaviridae*, genus *Alphavirus*, includes several major mosquito-borne pathogens. Alphaviruses can be categorized as Old or New World viruses, which are generally associated with febrile illnesses with either arthralgic or encephalitic syndromes, respectively (*1,2*). Mayaro virus (MAYV), an exceptional arthralgic New World alphavirus, produces Mayaro fever, which has signs and symptoms similar to those of dengue fever, including an acute febrile illness of 3–5 days' duration, typically with headache, retro-orbital pain, arthralgia, myalgia, vomiting, diarrhea, and rashes (*3,4*). Previous work has shown that some patients have persistent, severe joint pains for up to 1 year (*5*).

MAYV has a single-strand, positive-sense RNA genome ≈11,700 nt in length. The first two thirds of the genome encodes 4 nonstructural proteins (NSP1–4) and the other one third encodes the structural proteins (capsid, envelope [E] 3, E2, 6K/TF, and E1). Previous phylogenetic studies using a fragment of the structural polyprotein open reading frame suggest that MAYV occurs in 2 distinct genotypes, D and L (*6*). Genotype D includes isolates from all countries where MAYV has been detected, and genotype L contains strains detected only in Brazil (*6*).

MAYV was first isolated from forest workers in Mayaro, Trinidad in 1954 (*7*). Since 1954, there have been sporadic outbreaks of Mayaro fever (*5,6,8–11*), but most have occurred in Brazil, with the exception of a small outbreak in Bolivia in 2007 with 12 reported cases. MAYV has been isolated or antibodies against the virus were detected in Brazil, Colombia, Ecuador, Peru, Surinam, Bolivia, French Guiana, and Trinidad (*12–19*). Human MAYV infections were also detected serologically in Venezuela in a family that spent a night within a forest area (*20*).

The MAYV enzootic transmission cycle is not fully characterized. Previous studies suggest that it circulates between canopy-dwelling mosquitoes of the genus *Haemagogus* and nonhuman primates (*6,21*). Consequently, MAYV human seropositivity is largely associated with forest workers and hunters (*18*). MAYV has the potential to cause large outbreaks, as demonstrated in Brazil, where ≈800 persons were affected (*10*). Furthermore, *Aedes aegypti* mosquitoes are moderately competent vectors (*22*), which suggests that an urban human–mosquito–human transmission cycle could emerge, as has occurred for dengue, chikungunya, and yellow fever viruses with similar enzootic forest cycles (*23*).

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In January 2010, an outbreak of Mayaro fever in Venezuela occurred in La Estación village, Portuguesa State. By June 4, a total of 77 cases were recorded, which represents one of the largest outbreaks detected in South America. To understand the origins, evolution, and ecoepidemiology of MAYV, we sequenced complete genomes of 6 strains isolated during this outbreak and 21 additional isolates, which represented the full known spectrum of MAYV genetic diversity. We performed robust phylogenetic analyses on our complete genome data and previously published partial E2–E1 sequences.

## Materials and Methods

### Study Site and Outbreak Details

The study protocol was approved by the Naval Medical Research Center and Naval Medical Research Unit No. 6 Institutional Review Boards (protocols NMRCD.2000.0006, 2010.0010, and NAMRU6.2012.0016). Approval was given in compliance with all applicable federal regulations governing the protection of human subjects.

La Estación village is located at the northwestern corner of the municipality of Ospino, within Portuguesa State, Venezuela (Figure 1). It is a rural village with a population of 9,538 persons (average age 26 years). In the first quarter of 2010, an outbreak of a febrile illness with arthralgic manifestations was detected. Active surveillance was initiated by the Ministry of Health environmental health team.

### Virus Isolation and Identification

Viruses were isolated from human serum samples and identified on Vero E6 cell monolayers as described (*16*) by using a panel of polyclonal antibodies against alphaviruses and flaviviruses, followed by a MAYV-specific monoclonal antibody (MIAF TRVL4675). Six viruses were isolated from acute-phase serum samples of symptomatic patients. Signs and symptoms and other patient details are described in the results.

### Virus Propagation, Reverse Transcription PCR Amplification, and Sequencing

The 6 virus isolates from Venezuela were sequenced by using the Illumina HiSeq1000 platform (Illumina Inc., San Diego, CA, USA) as described (*24*). To facilitate a more detailed phylogenetic comparison, we determined complete genome sequences for 21 additional MAYV isolates from the World Reference Center for Emerging Viruses and Arboviruses Collection at the University of Texas Medical Branch (Galveston, TX, USA) (Table 1). Viruses were propagated once in Vero cells, and RNA was extracted from cell culture supernatant by using TriZol LS (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's recommendations. Complete genome sequences were obtained by using reverse transcription PCR (RT-PCR) amplification and sequencing of 6 overlapping RT-PCR amplicons (primer sequences available upon request). RT-PCRs were performed by using the Titan One-Step RT-PCR Kit (Roche Diagnostics, Indianapolis, IN, USA). PCR amplicons were visualized, excised, purified, and

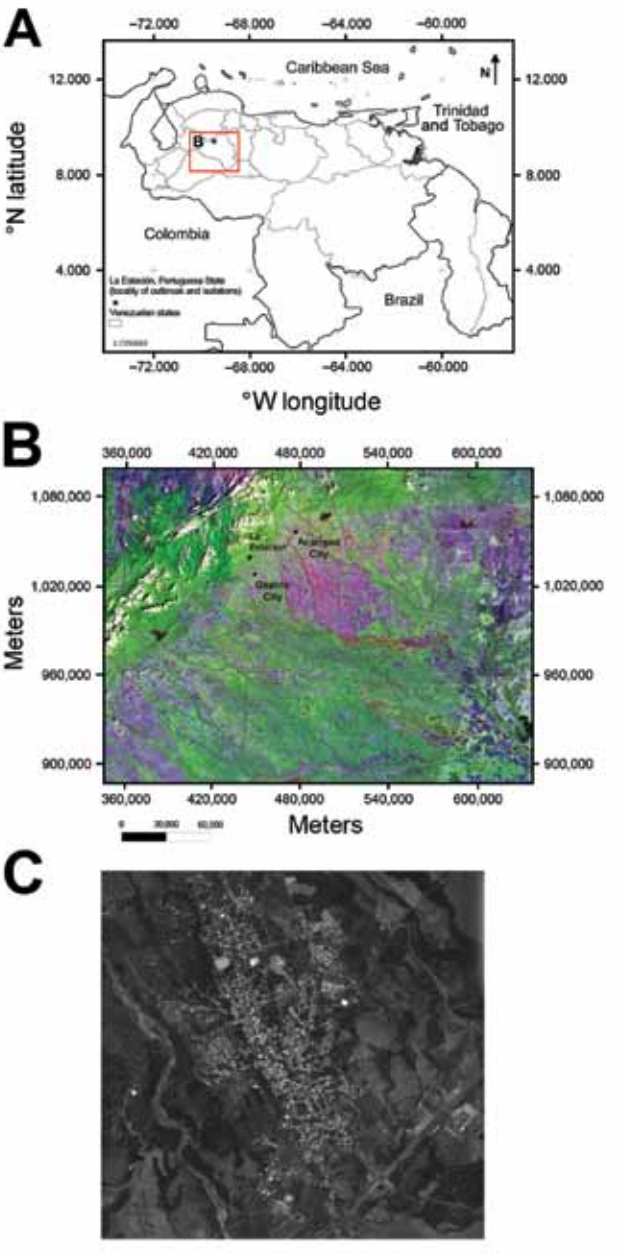


**Figure 1.** A) Location (red box) of outbreak of Mayaro virus, La Estación village, municipality of Ospino, Portuguesa State, Venezuela, 2010. Scale bar is at the lower left. B) Landsat image of eastern Andes and plains (Llanos) showing topography in Portuguesa State, Ospino, and La Estación, 28.5-m scale (http://glcf.umd.edu/data/landsat/). C) Spot image TM-5, 2.5-m scale, from La Estación, showing forest areas surrounding the urban–rural village (http://www.fii.gob.ve/proyectsFlags.html?value = 5).

**Table 1.** Characteristics of Mayaro virus strains sequenced, Venezuela, 2010*

| Isolate ID code | Source | Location | Year of collection | Genotype | GenBank accession no. |
|---|---|---|---|---|---|
| 11A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842795 |
| 12A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842796 |
| 13A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842797 |
| 14A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842798 |
| 15A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842799 |
| 16A | Human | La Estación, Portuguesa, Venezuela | 2010 | D | KP842794 |
| Ohio | Human | Loreto, Peru | 1996 | D | KP842807 |
| TRVL15337 | Mosquito | Trinidad | 1957 | D | KP842810 |
| BeH343148 | Human | Para, Brazil | 1978 | D | KP842803 |
| BeH186258 | Human | Brazil | 1970 | D | KP842809 |
| IQU3056 | Human | Loreto, Peru | 2000 | D | KP842808 |
| FSB1131 | Human | Bolivia | 2006 | D | KP842806 |
| IQE2777 | Human | Loreto, Peru | 2006 | D | KP842801 |
| BeAn337622 | Monkey | Para, Brazil | 1978 | D | KP842804 |
| FSB0319 | Human | Bolivia | 2002 | D | KP842805 |
| ARV0565 | Human | San Martin, Peru | 1995 | D | KP842800 |
| BeAn343102 | Monkey | Para, Brazil | 1978 | D | KP842802 |
| FMD0641 | Human | Puerto Maldonado, Peru | 2005 | D | KP842811 |
| FPY0046 | Human | Yurimaguas, Peru | 2011 | D | KP842813 |
| FVB0112 | Human | Bolivia | 2006 | D | KP842814 |
| FPI1761 | Human | Iquitos, Peru | 2011 | D | KP842815 |
| FPI0179 | Human | Iquitos, Peru | 2011 | D | KP842816 |
| FVB0069 | Human | Bolivia | 2006 | D | KP842817 |
| FMD3213 | Human | Puerto Maldonado, Peru | 2010 | N | KP842812 |
| BeH256 | Human | Para, Brazil | 1955 | L | KP842819 |
| BeAr30853 | Tick | Para, Brazil | 1961 | L | KP842820 |
| BeAr505411 | Mosquito | Para, Brazil | 1991 | L | KP842818 |

*ID, identification.

sequenced as described (*25*). Sequences were submitted to GenBank under accession nos. KP842794–KP842820.

### Sequence Analysis

Nucleotide sequences were aligned with MAYV sequences available in GenBank by using Clustal X (http://bips.u-strasbg.fr/fr/Documentation/ClustalX/), and manually adjusted by using Se-Al (http://tree.bio.ed.ac.uk/software/seal/). Twenty-nine genomic sequences (i.e., 27 determined in this study and 2 obtained from GenBank) were manually aligned, and untranslated terminal sequences were removed. A second dataset consisting of all partial E2–E1 envelope glycoprotein sequences (n = 68) was also analyzed. All sequences were confirmed as being nonrecombinant by using Recombination Detection Program version 4 (*26*).

The presence and nature of selective pressures acting on the MAYV genome were assessed by using methods available in Datamonkey (*27*), including the single-likelihood ancestor counting (SLAC) and internal fixed effects likelihood (IFEL) methods. Positive and negative selection events at each codon were determined.

### Phylogenetic Analysis

Maximum-likelihood (ML) phylogenetic trees were constructed by using the best-fit general time reversible + gamma 4 + invariable sites model, which was identified by using MODELTEST version 3.7 (*28*). Bootstrapping was performed to assess robustness of topologies by using 1,000 replicate neighbor-joining trees under the ML substitution model. Analyses were performed with PAUP* version 4.0b (Sinauer Associates, Inc., Sunderland, MA, USA).

### Coalescent Analysis

Bayesian coalescent analyses were performed by using a general time reversible + gamma 4 nucleotide substitution model, an uncorrelated lognormal molecular clock model, and a Bayesian Skyline population growth model. To ensure statistical efficiency, we applied a Bayesian stochastic search variable selection procedure (*29*). Inferences were obtained by using a Bayesian Markov chain Monte Carlo approach (*30*) run for 100 million generations with a 10% burn-in period and sampling every 10,000 states. Tracer version 1.5 (http://tree.bio.ed.ac.uk/software/tracer/) was used to monitor stationarity and efficient mixing. TreeAnnotator version 1.8.0 (http://beast.bio.ed.ac.uk) was used to summarize the posterior tree distribution, and FigTree version 1.3.1 (http://beast.bio.ed.ac.uk) was used to visualize the annotated maximum clade credibility (MCC) tree.

## Results

### Outbreak Investigation and Virus Isolation

During January–June 4, 2010, a total of 77 clinical cases compatible with MAYV infection were reported from La Estación. Fifty (65%) were in female patients and 27 (35%) in male patients; >50% of patients were homemakers

and laborers, and 38 (49%) were 25–54 years of age (*31*). MAYV was isolated from 6 of 19 acute-phase serum samples, all obtained from symptomatic case-patients. Signs and symptoms for these 6 patients (3 male patients and 3 female patients; age range 15–73 years) are shown in Table 2. Although all 6 patients had arthralgia, several did not have all characteristic signs and symptoms, such as headache (3/6) and nausea and vomiting (4/6). One case-patient (a 73-year-old woman) did not have fever.

### Sequence Divergence and Phylogenetic Analysis

In addition to sequencing the complete genomes of the 6 outbreak strains from Venezuela, we also sequenced complete genomes for 21 strains representing the full spectrum of genetic diversity, and known temporal and spatial distributions of MAYV (Table 1). Percentage nucleotide and amino acid sequence identities relative to outbreak strain 16A across all 9 genes of the genome for 10 strains that represent the full spectrum of genetic diversity determined for MAYV in this study are shown in Table 3. Analysis of nucleotide and amino acid sequence identities showed that MAYV is highly conserved (nucleotide sequence identities 96.4%–100% and amino acid sequence identities 97.7%–100%) among genotype D strains (Table 3). Comparison of genotype L with genotype D showed greater divergence (nucleotide sequence identities 83.8%–88.6% and amino acid sequence identities 90.9%–97.4%) for all genes.

An ML phylogeny based on the complete genome sequences of 29 MAYV strains and with a topology similar to that reported by Powers et al. (*6*) for partial E2–E1 sequences is shown in Figure 2. These results suggested that the trees based on partial genomes are sufficiently resolved for detailed coalescent analyses. A Bayesian MCC tree based on the partial E2–E1 fragment (nt 9,412–11,139 for DQ001069) from 68 sequenced MAYV strains, and with posterior probabilities indicated at relevant nodes is shown in Figure 3. The color of each lineage represents the most probable geographic location for the hypothetical ancestor at the node representing it. This phylogeny was consistent with the complete genome tree (Figure 2) and the phylogeny of Powers et al. (*6*). In addition to the previously described distinction between genotype D and L strains, there was some evidence of geographic structure within genotype D (Figure 3). In both our ML and MCC phylogenies, the 2010 outbreak sequences from Venezuela grouped within genotype D (posterior probability $\geq$0.99), and were most closely related to strains from Peru.

In addition to the previously described genotypes, a recently isolated (2010) strain from Peru (FMD3213) was intermediate between genotypes D and L and showed strong statistical support in both phylogenies. Given its year of isolation, strong support for its position in both phylogenies, and intermediary genetic distance from both genotypes D and L (Table 3), this strain might represent a previously undetected genotype, which we designate N.

### Selection Analysis

A mean global nonsynonymous:synonymous (dN:dS) ratio of 0.057 calculated by using the SLAC algorithm indicated that, similar to other arboviruses, purifying selection is the predominant evolutionary force driving MAYV evolution. This result was supported by the 274 codons found by SLAC and the 539 sites detected by IFEL to be under purifying selection. One codon was identified as being under positive selection by using SLAC and 5 by using IFEL ($p<0.1$). None of the 5 sites detected by IFEL delineated the 2010 outbreak strains, but these 5 sites instead primarily defined genotype L.

### Evolutionary Rates and Dates of Divergence

The mean evolutionary rate estimated from the genomic MAYV dataset was $1.67 \times 10^{-4}$ substitutions/site/year (95% highest posterior density [HPD] $1.02 \times 10^{-4}$–$2.41 \times 10^{-4}$). The rate for the clade containing the 2010 outbreak strains from Venezuela was estimated to be $1.34 \times 10^{-4}$ substitutions/site/year (95% HPD $0.8 \times 10^{-4}$–$1.9 \times 10^{-4}$) for partial E2–E1 sequences and $1.57 \times 10^{-4}$ substitutions/site/year (95% HPD $1.1 \times 10^{-4}$–$2.1 \times 10^{-4}$) for complete genome sequences. Rates for other MAYV lineages were $0.87 \times 10^{-4}$–$2.35 \times 10^{-4}$ substitutions/site/year for complete genome sequences and $0.75 \times 10^{-4}$–$2.40 \times 10^{-4}$ substitutions/site/year for partial E2–E1 sequences.

Estimated dates of divergence for selected lineages are shown in Table 4. The most recent common ances-

**Table 2.** Characteristics of 6 Mayaro virus–infected patients, Ospino, Venezuela, 2012*

| Strain ID code | Patient no. | Age, y/sex | Onset date† | Sampling date | Signs and symptoms | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Fever | Headache | Arthralgia | Nausea | Chills | Nasal congestion | Sore throat | Rash | Cough |
| 11A | 1 | 73/F | Feb 12 | Feb 14 | No | No | Yes | No | No | No | No | No | No |
| 12A | 2 | 38/F | Feb 12 | Feb 14 | Yes | Yes | Yes | Yes | No | No | No | No | No |
| 13A | 3 | 19/M | Feb 15 | Feb 19 | Yes | Yes | Yes | Yes | No | Yes | Yes | No | Yes |
| 14A | 4 | 46/F | Feb 18 | Feb 19 | Yes | No | Yes | No | Yes | Yes | Yes | Yes | No |
| 15A | 5 | 15/M | Feb 28 | Mar 1 | Yes | Yes | Yes | No | No | No | No | No | Yes |
| 16A | 6 | 47/M | Mar 13 | Mar 16 | Yes | No | Yes | No | Yes | No | No | No | No |

*ID, identification.
†Start date of signs and symptoms.

**Table 3.** Nucleotide and amino acid sequence identities among 9 major genes of the MAYV VZ2010 outbreak strain and 10 representative MAYV strains, Venezuela, 2010*

| | Strain | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Genotype D | | | | | | | | Genotype L | |
| MAYV VZ2010 gene | Ohio | FSB 1131 | ARV 0565 | BeAn 337622 | MAYLC | BeH 186258 | TRVL 15537 | FMD 3213 | BeH 256 | BeAr 505411 |
| *nsp1* | 99.4 (99.1) | 98.9 (99.1) | 98.6 (99.1) | 98.1 (98.7) | 98.4 (98.9) | 97.6 (99.1) | 97.3 (98.1) | 95.6 (98.7) | 88.6 (97.0) | 88.6 (97.0) |
| *nsp2* | 99.5 (99.7) | 98.7 (99.5) | 98.6 (99.6) | 98.4 (99.5) | 98.1 (99.1) | 96.8 (99.1) | 96.9 (98.9) | 93.7 (99.0) | 86.2 (97.4) | 86.2 (97.4) |
| *nsp3* | 99.7 (100.0) | 98.6 (100.0) | 97.8 (99.0) | 98.1 (99.2) | 97.7 (98.8) | 96.2 (98.5) | 96.7 (98.3) | 93.3 (78.4) | 82.7 (76.9) | 82.5 (76.9) |
| *nsp4* | 99.2 (100.0) | 98.3 (99.7) | 98 (99.8) | 97.8 (99.8) | 97.5 (99.8) | 97.1 (99.3) | 97.1 (99.2) | 92.5 (98.2) | 86 (97.2) | 86.2 (97.2) |
| C | 99 (100.0) | 98.7 (99.6) | 98.7 (100.0) | 98.1 (99.6) | 97.9 (100.0) | 96.5 (99.6) | 97.0 (100.0) | 93.5 (98.8) | 87.1 (96.1) | 88 (96.9) |
| E3 | 99.5 (100) | 98.5 (100.0) | 96.5 (100.0) | 97 (100.0) | 96.5 (100.0) | 97.5 (100.0) | 97.0 (100.0) | 93.4 (98.5) | 83.8 (90.9) | 84.8 (90.9) |
| E2 | 99.3 (99.8) | 98.4 (99.8) | 97.9 (99.1) | 98.3 (100.0) | 97.5 (99.5) | 96.1 (99.8) | 96.4 (98.8) | 93.3 (98.8) | 86.3 (97.4) | 86.2 (96.9) |
| 6K | 100 (100.0) | 98.3 (98.3) | 98.9 (100.0) | 98.3 (100) | 98.9 (100.0) | 98.3 (100.0) | 97.8 (100.0) | 97.8 (100.0) | 87.8 (95.0) | 88.3 (96.7) |
| E1 | 99.5 (99.8) | 99.1 (99.8) | 98.7 (99.5) | 98.5 (99.8) | 98.4 (99.8) | 97.9 (99.3) | 97.6 (97.7) | 94.3 (98.4) | 88 (96.1) | 87.6 (96.3) |

*Values in parentheses are amino acid identities. MAYV, Mayaro virus; *nsp*, nonstructural protein; C, capsid; E3, envelope small polypeptide; E2, envelope glycoprotein 2; 6K, envelope small polypeptide; E1, envelope glycoprotein 1.

tor (MRCA) was estimated to be 1864 (95% HPD 1822–1904) for genotype D and 1877 (95% HPD 1836–1915) for genotype L on the basis of partial E2–E1 data. All dates estimated within genotype D were strongly supported by the results based on complete genomes (Table 4). For example, the MRCA for 2010 isolates from Venezuela was estimated to have occurred during 2003–2009 (95% HPD) and have a mean estimate of 2007 based on the partial sequences. The estimate based on complete genomes was 2008 and had a smaller 95% HPD (2007–2009). The 95% HPDs for the genotype L time of MRCA based on partial E2–E1 sequences were wider. The MRCA for the node containing genotypes N and D was estimated to be 1657–1833 (95% HPD).

## Discussion

MAYV is a major emerging pathogen in northern South America and causes sporadic outbreaks of arthralgic disease in the Brazilian Amazon and eastern Bolivia. To date, these outbreaks have been relatively small, except for the epidemic in Belterra, Brazil, in 1977–1978. However, antibody detection and virus isolation rates indicate that MAYV commonly infects persons residing near enzootic transmission foci, and high incidence rates have been detected by using clinical surveillance (*32*).

The Mayaro fever outbreak at La Estación in 2010 is noteworthy because, with the exception of a family found to be seropositive (*20*), it probably represents the first outbreak documented in Venezuela. Also, with 77 reported cases, it is one of the largest outbreaks ever described. The signs and symptoms recorded corresponded to an influenza-like illness with arthralgia in most cases. One case-patient, a woman who was confirmed to be MAYV positive, had persistent arthralgia 1 month after infection. Cases of Mayaro fever are grossly underestimated in South America because extensive overlap in signs and symptoms means they typically fall under the dengue umbrella. It is essential that countries in South America test for MAYV when patients have dengue-like illness that also involves arthralgia to determine the true incidence of MAYV infection.

A larger proportion (65%) of cases were in female patients during this outbreak. Previous studies in Brazil and Bolivia have shown no sex bias during outbreaks of Mayaro fever (*9,10*), but a recent clinic-based surveillance study in Bolivia and Peru demonstrated that male patients were more likely to be MAYV infected, probably because of occupational exposure (*32*). It is unclear why we observed the opposite sex bias during the outbreak in La Estación. Further information on occupational exposure at this location is necessary to better understand the demographics of this outbreak of Mayaro fever.

La Estación is located in a former tropical forest that has recently been converted for coffee production and other farming (*33*). Several monkey species (i.e., *Cebus olivaceus* and *Alouatta seniculus*) and competent MAYV vectors (i.e., *Haemagogus* mosquitoes) are present within this village (*33,34*), which suggests enzootic circulation near human residences and work locations, possibly enhanced by encroachment into forests by local residents. This conclusion is supported by our data, which indicate that 50% of female case-patients, including those with higher antibody titers, primarily performed home activities; 37.5% of seropositive male patients performed coffee agricultural activities; and 63% of all seropositive persons resided near the coffee plantation.

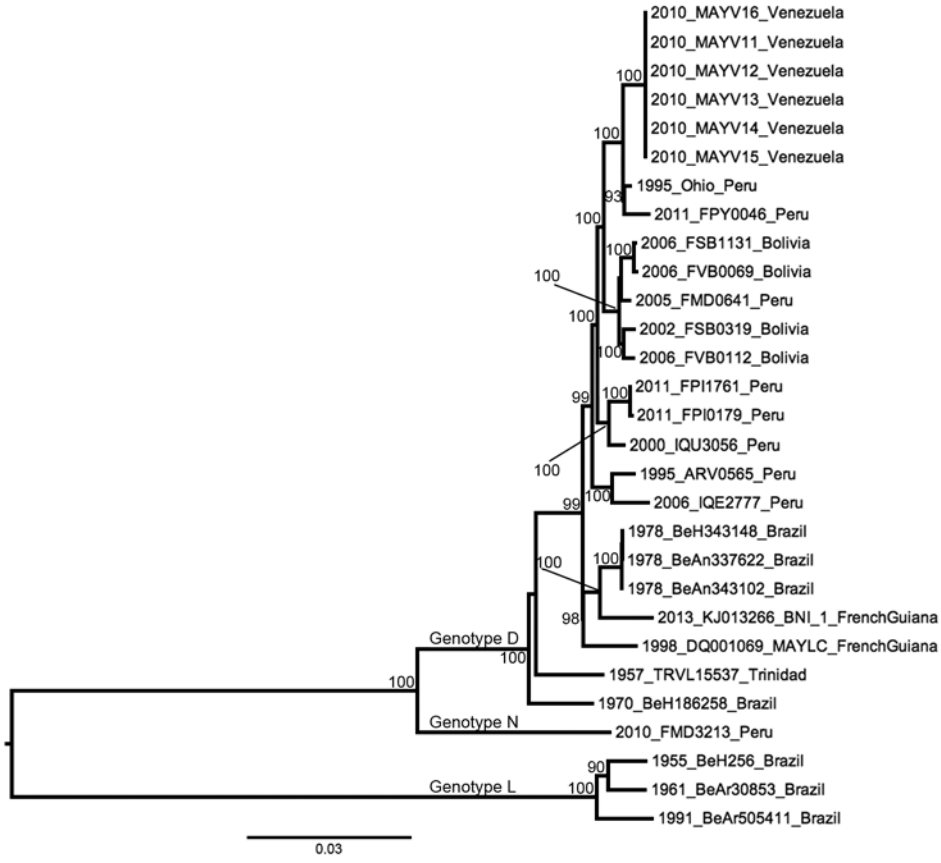


**Figure 2.** Midpoint-rooted maximum-likelihood phylogeny of 29 Mayaro virus strains on the basis of complete genome sequences, Venezuela, 2010. Nodes are labeled with bootstrap values ≥90%. Tip labels indicate year of isolation, strain name, and country of isolation. Scale bar indicates percentage nucleotide sequence divergence. Isolates DQ001069 and KJ013266 were previously sequenced and obtained from GenBank.

Nonsynonymous mutations that defined a specific group, clade, or lineage were identified manually. Uninformative mutations were not counted; only synapomorphies that were unique to a group or cluster were noted. There were 143 nonsynonymous synapomorphic mutations of interest, of which 114 were unique to genotype L. The 5 amino acid positions that were detected to be under positive selection and to delineate the genotype L strains were Leu→Ala/Val at position 518 in NSP1; Ala→Prol and Val→Thr at positions 298 and 386 in NSP3, respectively; Ala→Lys at position 249 in NSP4; and Leu→Thr at position 300 in the E1 protein.

In addition, strains from the outbreak in Venezuela in 2010 were also defined by 5 nonsynonymous mutations: Ser→Gly and Pro→Leu at positions 487 and 523 in NSP1, respectively; Val→Ile and His→Tyr at position 586 and 665 in NSP2, respectively; and Ile→Leu at position 156 in the E1 protein. However, these mutations were not identified as being subject to positive selection. Also, none of these unique mutations is in a genomic region known to affect the virulence or transmissibility of alphaviruses, and these amino acid substitutions are mostly conservative in nature. Whether these substitutions played any role in the emergence of MAYV is unclear. Reverse genetic studies are needed to determine if any of these substitutions cause major phenotypic changes.

In addition to the previously described MAYV genotypes (*6*), we detected a new genotype N that is genetically distinct and has strong phylogenetic support. We also further delineated several clades that segregated by geographic region (Figure 3). The estimate for the origin of MAYV strains in South America (i.e., 670 years [95% HPD 441–912 years] before 2013) should be interpreted with caution because a recent study of Venezuelan equine encephalitis complex alphaviruses showed that time of MRCA estimates for ancestral nodes dating more than a few hundred years ago is influenced by internal branch compression resulting from strong purifying selection (N.L. Forrester et al., unpub. data). Our coalescent analyses also estimated that genotypes D and L diverged ≈107 150 years before 2013, which suggests a relatively recent origin for these genotypes.

The apparent restriction of genotype L to Brazil, when compared with the wider distribution of genotype D, suggests geographic constraints on MAYV dispersal within Brazil. Because there is no apparent geographic barrier that can account for this distribution, the lack of genotype L strains from other countries might represent sampling bias, rather than a true population subdivision. However, we cannot exclude the possibility that potential restrictions associated with vector competence, vector distributions, or

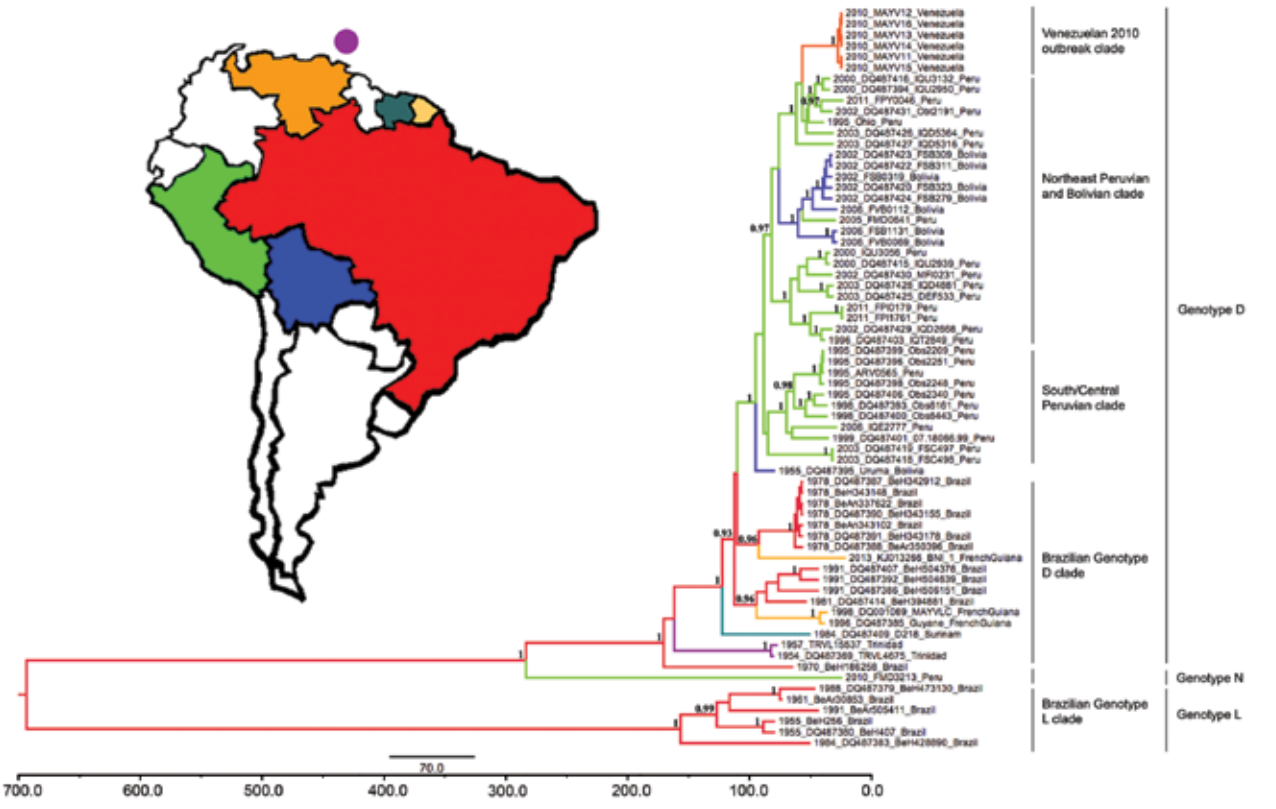


**Figure 3.** Bayesian maximum clade credibility tree for Mayaro virus in the Americas on the basis of 1,731 nt of envelope (E2–E1) genes, Venezuela, 2010. The purple circle above Venezuela indicates the island of Trinidad. Taxon labels indicate year of isolation, strain designation, and country of isolation. Terminal branches of the tree are colored according to the sampled location of the taxon at the tip. Internal branches are colored according to the most probable (modal) location of their parental nodes. Nodes with posterior probabilities (clade credibilities) ≥0.90 are indicated in black. Scale bar indicates time in years.

alternative vertebrate amplification hosts might affect this apparent population subdivision. The 6 virus sequences from the outbreak in Venezuela in 2010 (the only representatives from Venezuela) grouped as a monophyletic clade within genotype D and showed strong support as determined by genomic and partial sequences. Because genotype D strains show genetic diversity derived predominantly from viruses in Peru, it is not surprising that strains from Peru occupied positions basal to strains from the outbreak in Venezuela in 2010. Although there was strong support for the ancestral strain to have been derived from strains in Peru in 2010, this finding might also have resulted from sampling bias. There is a dearth of MAYV isolates and sequences from several intervening countries, including Brazil, Ecuador, and Colombia, and recent virus sequences from these countries are needed to identify whether MAYV is maintained continuously in Venezuela or if virus spread influences emergence. For example, Ecuador and Colombia might form the ecologic bridge between strains derived from Venezuela and the ancestral strains from Peru. Persons with antibodies against MAYV have been reported among Ecuadorian soldiers living in the Amazon (*18*), which supports this hypothesis.

Recent work on alphaviruses from Venezuela showed that a 1999 MAYV sequence grouped most closely with sequences from Trinidad in the Brazilian genotype D clade (*35*). However, these results were based only on E1 3′-untranslated region sequences, and their sequences could not be included in our analyses. Given the position of the 2010 sequences from Venezuela in our phylogeny, it appears that the 2010 outbreak strains did not descend from MAYV isolates previously circulating in Venezuela (i.e., at least since 1999). However, it is also possible that there is co-circulation or regionally independent evolution of genetically distinct MAYV strains within Venezuela. Yellow fever virus has a similar enzootic transmission cycle and was recently shown to undergo regionally independent evolution in Venezuela (*36*), which supports this hypothesis.

Analysis of sequence identities among MAYV genes demonstrated a high degree of conservation, similar to that seen for North American eastern equine encephalitis virus (*37*) and western equine encephalitis virus (*38*). These alphaviruses have relatively recent estimated times of MRCAs, occupy specific and similar ecologic niches, and appear to undergo continuous evolution without major subdivision by

**Table 4.** Estimated dates of divergence derived from partial E2 and complete genome sequences for major Mayaro virus lineages, Venezuela, 2010*

| Lineage | Date for time of MRCA for partial E2–E1 sequences (95% HPD) | Date for time of MRCA for complete genome sequences (95% HPD) |
|---|---|---|
| Venezuelan outbreak strains | 2007 (2003–2009) | 2008 (2007–2009) |
| Northeast Peruvian and Bolivian clade | 1952 (1935–1967) | 1966 (1940–1970) |
| South/Central Peruvian clade | 1968 (1953–1980) | 1977 (1967–1987) |
| Brazilian genotype D clade | 1864 (1822–1904) | 1872 (1836–1906) |
| Brazilian genotype L clade | 1877 (1836–1915) | 1905 (1882–1926) |
| Peruvian genotype N clade | 1750 (1657–1833) | 1746 (1658–1823) |

*E, envelope; MRCA, most recent common ancestor; HPD, highest posterior density.

geography or time. The MAYV tree topologies we observed also suggested continuous circulation within a distinct ecologic niche in South America. Eastern and Western equine encephalitis viruses have avian hosts, which might account for the lack of population subdivision by geography. In contrast, yellow fever virus, which is maintained by nonhuman primates, has a more geographically structured phylogeny (*39*). Despite the observation of high seroprevalence in nonhuman primates and isolation of MAYV from primatophilic mosquitoes (*6,10*), our tree topology and genetic conservation in MAYV suggest that birds or other highly mobile hosts might play some role in its dispersal. This finding could account for reduced geographic structure and observed branching patterns in MAYV phylogeny. Previous work has shown that MAYV replicates efficiently in avian cell cultures, such as Peking duck kidney cells and chick embryonic fibroblasts (*40*), and can achieve titers as high as 5.7–7 logs, suggesting that birds could be suitable reservoir hosts. However, further studies are necessary to determine if MAYV can replicate at the increased temperatures (≈43°C) in birds during levels of high activity. We hypothesize that during epizootics/epidemics, nonhuman primates are spillover hosts that might be especially susceptible to MAYV infection. The inability to continuously isolate or detect MAYV from *Haemagogus* spp. mosquitoes and nonhuman primate hosts in disease-endemic areas is consistent with the hypothesis that MAYV is maintained in a transmission cycle involving other vertebrate reservoir hosts.

This outbreak in Venezuela indicates that MAYV is a major emerging pathogen in South America, where persons residing near enzootic transmission foci might be at increased risk because of anthropogenic incursions. Strains from the outbreak in Venezuela in 2010 belong to genotype D and are distinct in the MAYV phylogeny. The single isolate from Puerto Maldonado in the Madre de Dios region of Peru in 2010 represents the only genotype N strain. Further surveillance at this and neighboring locations would be useful to determine the true extent of the genetic diversity of genotype N. Filling the current gaps in sequence data for geographic and temporal distributions of MAYV is needed to increase the phylogenetic resolution and aid our understanding of the evolution and spread of this emerging arthralgic alphavirus in the Americas.

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# Epidemiology of Lyme Disease, Nova Scotia, Canada, 2002–2013

**Todd F. Hatchette, B. Lynn Johnston, Emily Schleihauf, Angela Mask, David Haldane, Michael Drebot, Maureen Baikie, Teri J. Cole, Sarah Fleming, Richard Gould, Robbin Lindsay**

*Ixodes scapularis* ticks, which transmit *Borrelia burgdorferi*, the causative agent of Lyme disease (LD), are endemic to at least 6 regions of Nova Scotia, Canada. To assess the epidemiology and prevalence of LD in Nova Scotia, we analyzed data from 329 persons with LD reported in Nova Scotia during 2002–2013. Most patients reported symptoms of early localized infection with rash (89.7%), influenza-like illness (69.6%), or both; clinician-diagnosed erythema migrans was documented for 53.2%. In a separate serosurvey, of 1,855 serum samples screened for antibodies to *B. burgdorferi*, 2 were borderline positive (both with an indeterminate IgG on Western blot), resulting in an estimated seroprevalence of 0.14% (95% CI 0.02%–0.51%). Although LD incidence in Nova Scotia has risen sharply since 2002 and is the highest in Canada (16/100,000 population in 2013), the estimated number of residents with evidence of infection is low, and risk is localized to currently identified LD-endemic regions.

Lyme disease (LD) is an emerging vector-borne infection caused by *Borrelia burgdorferi*, which is transmitted to humans by infected ticks. In Nova Scotia, Canada, the vector is the blacklegged tick, *Ixodes scapularis*. Approximately 300,000 cases of LD occur in the United States each year (*1*). In Canada, infected *Ixodes* ticks are now endemic to parts of British Columbia (*I. pacificus*), Manitoba, Ontario, Quebec, New Brunswick, and Nova Scotia (*I. scapularis*) (*2*). The number of Canadians with LD has risen since LD became nationally reportable in 2009; a total of 682 cases from across the country were reported to the Public Health Agency of Canada (PHAC) in 2013, which most likely underrepresents the true number (*2–4*). As the geographic range of *I. scapularis* ticks expands, more Canadians will be at risk for LD.

Since the first case of locally acquired LD was reported in 2002, the number of human infections in Nova Scotia has risen sharply; 154 cases were reported in 2013 alone. LD can manifest with localized disease, most commonly erythema migrans (EM), or disseminated illness with neurologic, cardiac, and/or joint involvement. In the United States, up to 7% of cases are asymptomatic (*5*). In Canada, tick surveillance, coordinated and conducted by the Nova Scotia Departments of Health and Wellness and Natural Resources and the National Microbiology Laboratory (NML) of the PHAC, has identified the establishment of infected blacklegged tick populations in 6 regions in Nova Scotia, and these ticks have been found sporadically in many other locations, suggesting potential LD risk across the province. However, contemporary LD risk has been difficult to quantify in Nova Scotia because of the dynamic and expanding nature of vector tick populations, occurrence of missed and/or asymptomatic infections, and changes in surveillance methods and case definitions. Here we describe the epidemiology of LD and the results of a 2012 provincial serosurvey for antibodies to *B. burgdorferi* to characterize the features and estimate the risk for LD in Nova Scotia.

## Methods

### LD Case Data

LD is reportable in Nova Scotia. Case data were provided by Population Health Assessment and Surveillance, Nova Scotia Department of Health and Wellness (Table 1). Public health nurses investigate all LD cases by follow-up with the health care provider and patient. Data presented were extracted from case report forms. Numbers of tests conducted by the Capital District Health Authority Division of Microbiology, the Nova Scotia testing site for LD, were extracted from the laboratory information system. Regions to which LD is endemic (endemic regions) are defined by using the Canadian national definition. A confirmed endemic region is one in which active field surveillance has identified a reproducing population of ticks confirmed by the presence of all 3 stages on resident animals or in the

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**Table 1.** LD case definitions used in Nova Scotia, Canada*

| Period | Case classification | Case definition |
|---|---|---|
| 2002–2007 | Confirmed | EM or other clinical evidence and laboratory evidence of infection (based on CDC criteria) (*8*) |
| 2008–2015 | Confirmed | Clinical evidence of illness with a history of residence in, or visit to, an endemic region and laboratory evidence of infection (*8*) |
| 2008–2015 | Probable | Clinical evidence of illness without a history of residence in, or visit to, an endemic region and laboratory evidence of infection (*8*) OR clinician-observed EM without laboratory evidence but with history of residence in, or visit to, an endemic region |

*CDC, Centers for Disease Control and Prevention; EM, erythema migrans; LD, Lyme disease.

environment for at least 2 consecutive years, and *B. burgdorferi* is detected in these ticks and/or wild animal hosts in the locality (*2,6*). For the last 2 regions identified, all 3 stages were identified in 1 year only.

### Serosurvey

We used samples of residual serum from specimens submitted for diagnostic testing that would otherwise have been discarded. Aliquots of residual serum submitted for prenatal screening, electrolyte testing, cholesterol testing, or HIV screening were collected from regional laboratories during May 1–August 30, 2012. Each serum sample was deidentified so that it could not be linked to a specific patient. These specimens were chosen with the intent of obtaining samples from healthy persons undergoing blood tests as part of a regular health check-up. Serum samples were stratified by patient age, sex, and District Health Authority (DHA), and sampling was proportionate to the Nova Scotia population in five 10-year age groups for ages 10–59 years and 1 age group for ages 60–64 years. The Research Ethics Board of each DHA approved the serosurvey. One Research Ethics Board required an opt-out option for patients undergoing blood collection during the study period, achieved by publicizing the study using posters and asking patients who did not want their serum used to identify themselves at the time of collection. No patients opted out.

### Serologic Testing

We detected antibodies to *B. burgdorferi* using a commercially available enzyme immunoassay (EIA) that used a whole-cell sonicate of *B. burgdorferi* (*B. burgdorferi* ELISA II, Wampole Laboratories, Princeton, NJ, USA). Samples were tested at the Capital DHA microbiology laboratory. In accordance with Canadian and US guidelines (*7*,*8*), specimens that screened positive or equivocal on the EIA were sent to the NML for confirmatory testing. The NML uses a 2-tiered approach whereby specimens that test positive or equivocal by EIA are retested by using C6 ELISA (Immunetics, Boston, MA, USA) and Western blot (Euroimmun, Morris Plains, NJ, USA). Consistent with criteria from the US Centers for Disease Control and Prevention, a positive IgG Western blot (WB) (5 of 10 significant bands reactive) was considered conclusive evidence of previous infection (*9*). In addition, the NML has created a borderline category for serum for which 4 of 10 reactive significant bands are documented but includes an additional fifth band that is visible on the blot but is insufficiently intense to be considered reactive.

### Statistics

We calculated sample size for the serosurvey on the basis of an estimated seroprevalence of 1.0% ± 0.5% precision within a 95% CI, with oversampling in DHA 1, DHA 2, and DHA 3, where greater LD activity has been noted (Figure 1). Descriptive statistics were used to characterize reported LD cases and testing results from the serosurvey. Seroprevalence estimates were produced and 95% CIs calculated by using the Clopper-Pearson Exact method. Design weights accounted for regional oversampling in the provincial estimates. Statistical analyses were conducted by using SAS version 9.4 (SAS Institute, Inc., Cary, NC, USA). Maps were created by using QGIS 2.0 (http://qgis.org/en/site/). Population estimates were based on 2011 census data from Statistics Canada (*10*).

## Results

### Endemic Regions

The first established *B. burgdorferi*–infected blacklegged tick population in Nova Scotia was identified in a rural region within DHA 1 in 2003. The second endemic region was identified in a park within the largest urban center in DHA 9 in 2006. In 2008, a rural region within DHA 2 was declared to be endemic, and in 2010, a rural region in DHA 6 was declared endemic. In 2011 and 2012 the fifth and sixth endemic regions were identified in other rural regions within DHAs 1 and 2, >20 km away from regions previously identified.

### LD Cases

A total of 329 LD cases were reported in Nova Scotia during 2002–2013. Case-patients were a median of 56 (range 0–85) years of age, and most (76.9%) were male (Table 2). Only 26.4% of LD patients reported a definite history of a tick bite. Most reported symptoms of early localized LD, including influenza-like illness and EM or other non-EM rash. Physician-diagnosed EM was reported for 53.2% of cases. Central clearing (i.e., reduction or of the erythema near the center of the rash) was reported for 49.7%; a total

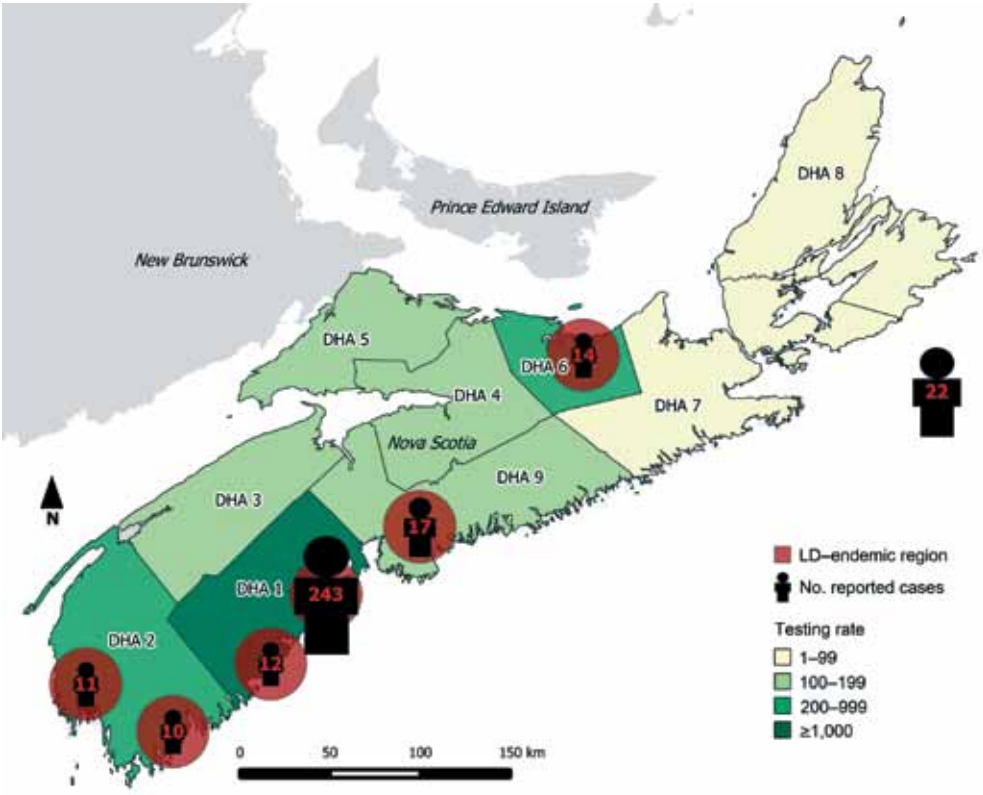


**Figure 1.** Reported cases of Lyme disease (LD) for 2002–2013, by endemic region of exposure and LD testing rates by District Health Authority (DHA) for 2013, Nova Scotia, Canada. Of the 22 cases without a known link to an LD-endemic area in Nova Scotia, 17 persons were infected outside the province (Europe and the United States); for 5 persons, location of exposure was either unknown or outside of known LD-endemic regions. Testing rate is per 100,000 population.

of 33.7% reported no central clearing, and for 16.6%, central clearing was unknown. A total of 125 LD patients had early disseminated or late infection, including recurrent joint swelling and Bell palsy. The percentage of cases with clinician-observed EM increased over time, and brief recurrent joint swelling was reported in a greater percentage of patients in 2013 than previously (Figure 2). Thirteen LD patients were hospitalized (Table 3), but the reasons for admission were unknown.

A total of 307 (93%) case-patients reported living in or traveling to an endemic region in Nova Scotia (Figure 1). Of the remaining case-patients, 17 were infected outside the province, including patients who traveled to Europe and the United States; for 5 case-patients, the location of exposure was either unknown or outside of regions where LD is known to be endemic. Most (73.3%) cases met the definition for a confirmed case. Most (71.4%) case-patients lived in DHA 1. The number of cases increased substantially during 2012–2013, attributable to increased reports of cases associated with known endemic regions (Figure 3).

A total of 263 (80%) cases had serologic evidence of infection. Of the 66 cases classified as "probable: EM + endemic exposure" that were reported to Nova Scotia public health professionals, 10 did not have any information about laboratory testing, 42 did not have serologic testing, and 14 had serologic test results that were negative by the 2-tier algorithm. Of these 14 patients, 3 were negative on the whole-cell EIA, and 11 were positive by the C6 ELISA but negative by WB.

The annual number of serologic tests for LD in Nova Scotia increased from 1,659 in 2010 to 2,421 in 2013. Testing rates varied by DHA (Figure 1), ranging from 69.1 tests per 100,000 population in DHA 8 to 1,581.9 per 100,000 population in DHA 1 in 2013. Serologic incidence of LD in 2013 varied by DHA, ranging from 0 cases per 100,000 population in DHA 8 to 206.2 per 100,000 population in DHA 1.

### Serosurvey Results

Of 1,855 serum samples tested for antibodies to *B. burgdorferi*, 215 (11.6%) screened positive by the whole-cell EIA (Walpole Laboratories, Walpole, MA, USA) and were sent to the NML for confirmatory testing. Of these, 17 (0.9% of total serosurvey) were positive or equivocal by the C6 ELISA. None were positive by IgG WB using Centers for Disease Control and Prevention (CDC) criteria, but 2 samples were borderline positive (Table 3). If we used CDC criteria for WB interpretation, the estimated seroprevalence of LD in Nova Scotia was 0% (95% CI 0.00%–0.20%). If we considered the 2 borderline WB results or the positive C6 ELISA as evidence of exposure to *B. burgdorferi*, the estimated seroprevalence was 0.14% (95% CI 0.02%–0.51%) and 0.98% (95% CI 0.56%-1.60%), respectively (Table 4).

## Discussion

LD is emerging in Nova Scotia, and public health surveillance data have been useful for characterizing risk and describing the clinical presentation of LD. Most cases are characterized by early localized disease. Compared with data from the United States, Nova Scotia case-patients were less likely to have EM reported as the presenting manifestation (69% vs. 53%) (*11*). Although published studies report that up to 80% of LD case-patients have EM, these findings were in the context of a vaccine trial or active laboratory-based surveillance study in a hyperendemic region where patients were followed closely and physicians were very experienced in diagnosing EM (*12*,*13*). The public health surveillance system in Nova Scotia captures both clinician-diagnosed EM and other skin rashes. Although almost 90% of case-patients reported a rash, only 53% had clinician-diagnosed EM. However, some of the other skin rash cases are also likely to represent EM. Central clearing in only 50% of EM lesions was consistent with other reports that EM might have diffuse erythema or enhanced central erythema rather than central clearing (*14*). An increase in the proportion of cases with clinician-diagnosed EM over time suggests physicians have become more aware of and/or better able to diagnose EM.

The most common manifestation of late LD in Nova Scotia was arthritis. Although up to 60% of untreated LD results in arthritis, earlier recognition and treatment is expected to greatly reduce its frequency (*7*,*15*–*17*). The increase in the proportion of Nova Scotia case-patients reporting recurrent brief episodes of large joint swelling to 32.5% in 2013 is similar to the proportion of US cases of Lyme arthritis (30%) (*11*). The higher proportion of cases with arthritis in 2013 does not necessarily reflect a large proportion of missed cases during earlier years but might be related to misreporting of arthralgia and reporting bias toward serologically confirmed cases, as well as a general increase in the number of cases and duration of LD in Nova Scotia. Further investigation is needed to understand how many case-patients reporting joint symptoms fit the clinical diagnosis of Lyme arthritis.

As in the United States, other manifestations, including atrioventricular block and neuroborreliosis, were

**Table 2.** Characteristics of 329 reported LD case-patients, Nova Scotia Canada, 2002–2013*

| Characteristic | No. (%) |
|---|---|
| Sex | |
| M | 200 (60.8) |
| F | 129 (39.2) |
| Age group, y | |
| 0–9 | 31 (9.4) |
| 10–19 | 33 (10.0) |
| 20–29 | 17 (5.2) |
| 30–39 | 17 (5.2) |
| 40–49 | 32 (9.7) |
| 50–59 | 67 (20.4) |
| 60–69 | 76 (23.1) |
| 70–79 | 44 (13.4) |
| ≥80 | 12 (3.6) |
| Case definition met | |
| Confirmed | 241 (73.3) |
| Probable | |
| EM with history of exposure to endemic region | 66 (20.1) |
| Clinical evidence of illness without exposure to endemic region with laboratory evidence | 22 (6.7) |
| Symptom† | |
| Rash, any reported | 295 (89.7) |
| Influenza-like illness | 229 (69.6) |
| EM, physician-diagnosed | 175 (53.2) |
| Recurrent brief episodes of large joint swelling | 77 (23.4) |
| Bell palsy | 23 (7.0) |
| Nervous system symptoms, excluding Bell palsy | 21 (6.4) |
| Cardiovascular system signs | 4 (1.2) |
| History of tick bite | |
| Definite | 87 (26.4) |
| Possible, exposure to wooded or brushy regions | 199 (60.5) |
| Unknown | 43 (13.1) |
| DHA of residence‡ | |
| DHA 1 | 235 (71.4) |
| DHA 2 | 21 (6.4) |
| DHA 3 | 9 (2.7) |
| DHA 4 | 4 (1.2) |
| DHA 5 | 1 (0.3) |
| DHA 6 | 8 (2.4) |
| DHA 7 | 4 (1.2) |
| DHA 8 | 2 (0.6) |
| DHA 9 | 45 (13.7) |
| Hospitalized | 13 (4.0) |

*Endemic regions as defined in June 2014. Some cases classified to exposure retrospectively (i.e., case occurred before endemic region were declared). DHA, District Health Authority; EM, erythema migrans; LD, Lyme disease.
†More than 1 symptom might be reported per case-patient. Rash, any reported, includes EM. Cardiovascular system signs include atrioventricular block, mycarditis, other.
‡DHA 1, endemic regions declared in 2003 and 2012; DHA 2, endemic regions declared in 2008 and 2011; DHA 6, endemic region declared in 2010; DHA 9, endemic region declared in 2006.

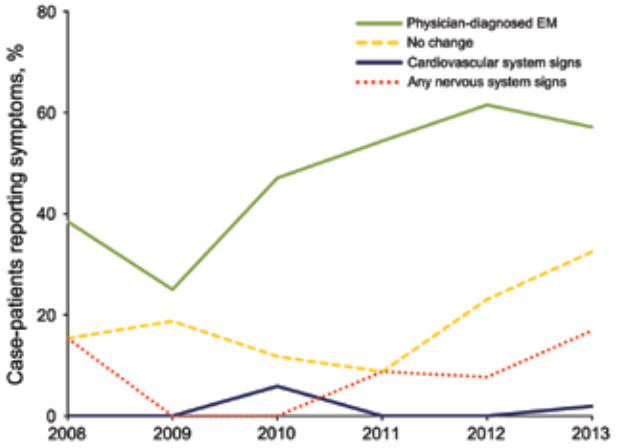


**Figure 2.** Percentage of Lyme disease (LD) case-patients with symptom complex, by year, Nova Scotia, Canada, 2008–2013. Cardiovascular system signs include atrioventricular block, mycarditis, and other. Nervous system signs comprise peripheral or central signs. EM, erythema migrans.

**Table 3.** Characteristics of reported LD in 13 hospitalized patients, Nova Scotia Canada, 2002–2013*

| Characteristic | No. (%) |
|---|---|
| Sex | |
| M | 10 (76.9) |
| F | 3 (23.1) |
| Age group, y | |
| 0–9 | 2 (15.4) |
| 10–19 | 2 (15.4) |
| 20–29 | 2 (15.4) |
| 30–39 | 2 (15.4) |
| 40–49 | 1 (7.7) |
| 50–59 | 1 (7.7) |
| 60–69 | 1 (7.7) |
| 70–79 | 1 (7.7) |
| ≥80 | 1 (7.7) |
| Symptoms† | |
| Rash, any reported | 4 (30.8) |
| Influenza-like illness | 9 (69.2) |
| EM, physician-diagnosed | 4 (30.8) |
| Brief recurrent joint swelling | 2 (15.4) |
| Bell palsy | 3 (23.1) |
| Any nervous system sign or symptom | 2 (15.4) |
| Cardiovascular system signs | 2 (15.4) |

*EM, erythema migrans; LD, Lyme disease.
†More than 1 symptom might be reported per case-patient. Rash, any reported, includes EM. Cardiovascular system signs include atrioventricular block, mycarditis, other.

uncommon (*11*). Only 26% of LD patients in Nova Scotia recalled a tick bite, consistent with US data where 84% and 86% of patients with EM and Lyme arthritis, respectively, failed to recall exposure to blacklegged ticks (*18*–*21*).

The incidence of LD in Canada has increased during the last decade (*2*,*4*), and Nova Scotia has the highest reported incidence (16 cases/100,000 population in 2013). Incidence varies greatly geographically; 1 health region (DHA 1) reported case rates of ≈200/100,000 population in 2013, which is higher than those in northeastern US states (*11*). The increased incidence most likely resulted from the increased number of established blacklegged tick populations; increases in the size, geographic range, and pathogen prevalence within these populations; increasing rates of human infection; and better identification and reporting of LD. Infected blacklegged ticks can be sporadically identified in other regions of the province, but only 1.5% of LD cases were not linked to known endemic regions, suggesting the risk remains localized. Our serosurvey did not identify any persons with *B. burgdorferi* antibodies based on CDC criteria, and even if the 2 borderline results were included, the estimated provincial seroprevalence was much less than 1%. Even in DHAs known to contain endemic foci of LD, we failed to detect any positive serum and found an estimated seroprevalence of <2% in the DHA with the highest reported LD incidence. *B. burgdorferi* seroprevalence in the northeastern United States, with similar environmental conditions to Nova Scotia, ranges from 0% to 18.8% (*22*,*23*). The fact that tick populations endemic to the northeastern United States have been established for much longer than in Nova Scotia might explain this difference. Widespread clinical testing, coupled with the serosurvey results, suggests LD cases most likely are not being missed in Nova Scotia.

Although all of the serum used for the serosurvey was negative by 2-tier testing, 8% of serum samples positive by whole-cell EIA (17/215) were positive on the C6 ELISA. Although some data suggest that classifying LD on the basis of C6 alone is more sensitive than the 2-tier algorithms (*24*), the potential exists for false-positive and false-negative results. The literature suggests that the C6 ELISA specificity is 98.4%–98.6% (*24*,*25*). However, when used as the supplemental test to positive whole-cell lysate EIA, as in our study, its specificity is estimated at 99.1%–99.8% (*26*). The 17 positive C6 results in our study could be in keeping with false-positive results, with this reported specificity. Alternatively, these reactive C6 serum samples

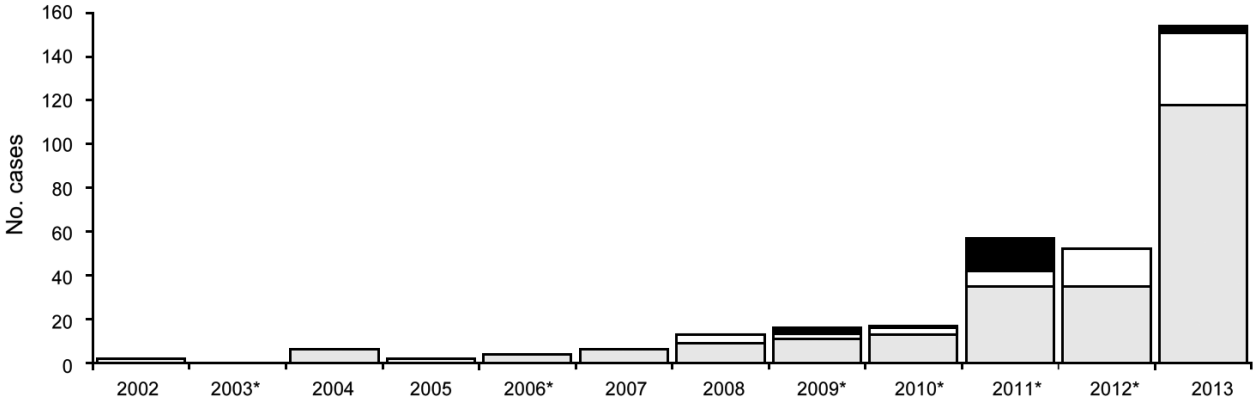


**Figure 3.** Number of reported Lyme disease (LD) cases, by case classification and year, Nova Scotia, Canada, 2008–2013. Black indicates probable cases—clinical illness and positive serology (2008–2013). White indicates probable cases—clinician-diagnosed erythema migrans and exposure to LD-endemic region (2008–2013). Light gray indicates confirmed case—erythema migrans or other clinical illness and positive serology (2002–2007); previous definition plus exposure to LD-endemic region (2008–2013). Asterisk indicates years when LD-endemic regions were declared.

**Table 4.** LD test results from serosurvey and seroprevalence estimates, Nova Scotia, Canada, 2002–2013*

| Region (no. endemic regions) | Laboratory test type | | | | Seroprevalence estimate | |
|---|---|---|---|---|---|---|
| | Total screening tests, whole-cell EIA | Whole-cell EIA positive or indeterminate, no. (%) | C6 positive or equivocal, no. (%) | IgG WB borderline, no. (%) | IgG WB borderline, % (95% CI) | C6 ELISA, %, (95% CI) |
| DHA 1 (2) | 191 | 21 (11.0) | 1 (0.5) | 0 | 0 (0–1.9) | 0.52 (0–2.9) |
| DHA 2 (2) | 199 | 19 (9.5) | 2 (1.0) | 0 | 0 (0–1.8) | 1.01 (0.1–3.6) |
| DHA 3 | 261 | 42 (16.1) | 2 (0.8) | 0 | 0 (0–1.4) | 0.77 (0.1–2.7) |
| DHA 4 | 120 | 12 (10.0) | 1 (0.8) | 1 (0.8) | 0.83 (0–4.6) | 0.83 (0–4.6) |
| DHA 5 | 44 | 1 (2.3) | 1 (2.3) | 0 | 0 (0–8.0) | 2.27 (0.1–12.0) |
| DHA 6 (1) | 74 | 3 (4.1) | 0 | 0 | 0 (0–4.9) | 0 (0–4.9) |
| DHA 7 | 72 | 3 (4.2) | 0 | 0 | 0 (0–5.0) | 0 (0–5.0) |
| DHA 8 | 201 | 22 (10.9) | 1 (0.5) | 0 | 0 (0–1.8) | 0.5 (0–2.7) |
| DHA 9 (1) | 693 | 92 (13.3) | 9 (1.3) | 1 (0.1) | 0.14 (0–0.8) | 1.3 (0.6–2.5) |
| Nova Scotia† | 1,855 | 215 (11.6) | 17 (0.9) | 2 (0.1) | 0.14 (0.02–0.51) | 0.98 (0.56–1.60) |

*DHA, District Health Authority; EIA, enzyme immunoassay; LD, Lyme disease; WB, Western blot.
†Provincial seroprevalence estimates weighted by age, sex, and DHA, accounting for oversampling in DHAs 1, 2, and 3.

could represent patients infected with *B. burgdorferi* and treated early with appropriate antimicrobial drugs. However, because the serum used could not be linked to patients, we have no clinical information to support this possibility. Data suggest that the C6 ELISA has a higher sensitivity for early infection and can be positive before the IgG WB completely matures to include the required 5/10 bands (*27*). In addition, evidence suggests that patients treated early in infection abort the seroconversion response, and a positive IgG WB might not develop (*27*,*28*). Another possibility is that the positive C6 ELISA results from cross-reactivity with another *Borrelia* species, such as *B. miyamotoi*, which has been identified in blacklegged ticks in the United States and Canada, including Nova Scotia (*29*,*30*). Using EIA and WB assays specific for *B. miyamotoi*, Krause et al. found serologic evidence of acute infection in patients living in endemic regions who had a viral-like illness (*31*). Although a recent study found 2 of 34 ticks from Nova Scotia submitted as part of passive surveillance had positive PCR for *B. miyamotoi* (*30*), no data are available that examined the potential cross-reactivity of whole-cell sonicate or C6 *B. burgdorferi* EIAs with patient serum containing antibodies to *B miyamotoi*. If these hypotheses are correct, we should have seen a disproportionate number of positive serum samples from regions with the highest risk for exposure to infected blacklegged ticks, such as DHA 1, where an infected population of blacklegged ticks has been established since 2003 and where most case-patients reside and were exposed. However, seroprevalence of C6 positive serum did not differ significantly among any of the DHAs.

Our study has several limitations. Our clinical data are limited to the case report forms used by Nova Scotia public health professionals; thus, the precision regarding the clinical presentation is limited. For example, the inclusion of categories of influenza-like illness is not specific and could represent a respiratory illness. Furthermore, the case reports do not always differentiate between single EM and multiple EM, so classification of early localized and early disseminated infection was not possible. Early LD, the most common presentation, is predominantly a clinical diagnosis for which sensitivity of serologic testing is poor (<50%) and not recommended. Thus, surveillance data might underestimate incidence if not all clinical cases are captured. We recognize that estimating the extent of underreporting is difficult and that underreporting could vary geographically. However, in the cohort reported here, 42 of 66 cases classified as "probable: EM + endemic exposure" followed the Infectious Diseases Society of America guidelines (*7*) and were not tested serologically. These reports have come from 5 different districts across the province suggesting that physicians are reporting at least some *B. burgdorferi* infections on the basis of clinical presentation alone.

Another limitation is that the serosurvey samples might not be representative of the population at risk. We used samples of residual serum from diagnostic testing that might be biased toward a population with more medical comorbidities or different risk and health-seeking behaviors (*32*). Our study attempted to reduce this bias by selecting samples that were originally collected for routine diagnostic testing, with the aim of capturing persons undergoing routine well-person screening. However, this sample will exclude persons who generally do not access regular medical care. Despite this limitation, the sampling method has been used for other infections and is thought to provide an acceptable balance between representativeness and feasibility (both practical and financial). In fact, the only published study that has compared the residual serum approach with population-based sampling yielded comparable estimates of immunity against 5 vaccine-preventable diseases, with an ≈7-fold increased cost for the population-based approach (*33*). Still, risk for exposure to blacklegged ticks through outdoor activity in endemic regions was unavailable for serosurvey specimens and probably has greater heterogeneity than probability of vaccination. Also, our sample did not include the 0–9- or ≥65-year age groups.

Although LD incidence is increasing in Nova Scotia, infections appear to be restricted to regions within the

province where populations of infected blacklegged ticks are known to be endemic. These findings support a targeted approach to public health risk messaging. Our seroprevalence study suggests that <1% of Nova Scotia residents have been exposed to *B. burgdorferi*. However, as tick populations continue to expand, we expect LD rates to continue to increase. Residents of, and travelers, to Nova Scotia need to be vigilant and take precautions to reduce their risk for LD when they venture into regions where ticks are present. Because only a minority of patients will report a tick bite, physicians should be aware of the manifestations of LD and consider it when patients have compatible symptoms and exposure to an endemic region, through residence or travel, in Nova Scotia.

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# Environmental Factors Related to Fungal Wound Contamination after Combat Trauma in Afghanistan, 2009–2011

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During the recent war in Afghanistan (2001–2014), invasive fungal wound infections (IFIs) among US combat casualties were associated with risk factors related to the mechanism and pattern of injury. Although previous studies recognized that IFI patients primarily sustained injuries in southern Afghanistan, environmental data were not examined. We compared environmental conditions of this region with those of an area in eastern Afghanistan that was not associated with observed IFIs after injury. A larger proportion of personnel injured in the south (61%) grew mold from wound cultures than those injured in the east (20%). In a multivariable analysis, the southern location, characterized by lower elevation, warmer temperatures, and greater isothermality, was independently associated with mold contamination of wounds. These environmental characteristics, along with known risk factors related to injury characteristics, may be useful in modeling the risk for IFIs after traumatic injury in other regions.

Trauma-related invasive fungal infections (IFIs) generally develop as a complication after a penetrating wound has been inoculated with fungal spores. Although trauma-related IFIs are not as common as bacterial infections, they are characterized by substantial morbidity (e.g., limb amputations) and mortality rates as high as 38% in civilian populations (*1*–*7*). Trauma-related IFIs have been reported in persons who sustained injuries in agricultural/industrial accidents, natural disasters, and combat (*2*–*12*).

One of the largest reported series of trauma-related IFIs occurred among military personnel who sustained combat-related injuries in Afghanistan (77 case-patients and an overall incidence rate of 6.8%) (*13*,*14*). In a multivariable analysis, the following risk factors were independently associated with the development of IFIs: blast injuries sustained while person was on foot patrol (dismounted), traumatic above-knee amputations, and super-massive (>20 units) transfusions of packed red blood cells during the first 24 hours after injury (*14*).

Although location was not included in the risk factor analysis in the previous study, clinicians have recognized that military personnel with IFIs predominantly sustained injuries in the southern provinces of Afghanistan (i.e., Helmand and Kandahar) (*6*,*14*). As with other regional infectious diseases, climate and environmental conditions may be key factors. Because of the high morbidity and mortality resulting from trauma-related IFIs, specifically identifying any potential factors associated with exposure is critical to mitigating risk by supporting timelier diagnosis and treatment. Therefore, our objectives were to assess traumatic wound mold contamination in regards to environmental conditions unique to injury circumstances for individual patients and also to investigate geographic and environmental factors associated with regional grouping of cases.

## Methods

### Study Population

The study population included 1,133 US military personnel who sustained combat-related injuries in Afghanistan from June 1, 2009, through August 31, 2011. After medical evacuation to Landstuhl Regional Medical Center in Landstuhl, Germany, the patients were transferred to 1 of 3 participating military hospitals in the United States: Walter Reed Army Medical Center (Washington, DC, USA), National Naval Medical Center (Bethesda, MD, USA), and Brooke Army Medical Center (San Antonio,

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TX, USA). Data from these patients were collected as part of a longitudinal, prospective cohort study which analyzed infectious complications among military personnel with deployment-related trauma, the US Department of Defense–Department of Veterans Affairs, Trauma Infectious Disease Outcomes Study (*15*). Characteristics and injury circumstances for patients who met criteria for inclusion in the analysis (i.e., injured during deployment, ≥18 years of age, evacuated from theater to Landstuhl Regional Medical Center, and transferred to a participating hospital in the United States) were collected from the Department of Defense Trauma Registry (*16*). The study received approval from the Infectious Disease Institutional Review Board of the Uniformed Services University of the Health Sciences (Bethesda, MD, USA).

### Case-Patient and Control-Patient Identification

In a prior nested case–control study within a cohort that examined risk factors for the development of an IFI after a combat-related injury, we identified 76 IFI case-patients from 1,133 wounded personnel using established IFI case definitions and matched them with 150 control-patients by injury severity score (±10) and date of injury (±3 months) (*14*). For the nested case–control study described here, we retrieved grid coordinates for the location of injury for patients who were part of the original case–control study from the US Department of Defense Joint Trauma Analysis and Prevention of Injury in Combat program (Fort Detrick, MD, USA) and imported the data to ArcGIS, a geographic information system (GIS) software package (http://www.esri.com). Because grid coordinates were not available for all patients included in the original analysis, the analysis herein incorporates data from a subset of the cohort (71 IFI case-patients and 101 control-patients). Although the original analysis involved matching, the analysis described here was an unmatched case–control analysis.

As previously stated, the observed IFI cases have been identified to cluster in the southern region of Afghanistan (Figure 1) (*6*,*17*). The subset of 172 patients with available grid coordinates were classified on the basis of the presence of mold wound contamination, whether or not the patient's condition progressed to an IFI. This classification is based on the wound microbiology results obtained at either Landstuhl Regional Medical Center or at hospitals in the United States. Specifically, the mold contamination group included patients with wound cultures that grew mold. If wound cultures did not grow mold, but the patient's wound had histopathologic features or angioinvasion that met the case definition of an IFI (*13*), the patient was included in the mold contamination group. By these criteria, all 71 IFI case-patients were identified as having fungal infections. Patients who had no mold growth and did not meet IFI case definitions were included in the noncontaminated group.

For our analysis, we selected 2 combat zones in Afghanistan (southern and eastern regions) based on casualty rates and compared the regions using environmental variables. The southern region included Helmand and Kandahar Provinces and the eastern region consisted of the area east and north of Zabul Province (Figure 1). Five patients injured outside the southern and eastern regions were excluded from the analysis.

### Environmental Data

Two digital datasets were used for a visual comparison with injury locations. Land cover data, originally developed by the Food and Agriculture Organization using 1992 satellite data, were downloaded from MapCruzin (http://www.mapcruzin.com). River data, originally part of the Digital Chart of the World dataset, were downloaded from DIVA-GIS (http://www.diva-gis.org.

For the statistical analysis and modeling, we obtained several raster environmental datasets and imported them to ArcGIS version 10.2; using the "Extract Values to Points" option, the values of the pixels at injury locations were copied from each environmental layer and recorded in a table for further analysis. We obtained 1-km bioclimatic raster data from WorldClim (http://www.worldclim.org); variables included datasets such as precipitation of the wettest month and minimum temperature of the coldest month of the year. These variables are derived from averages of 50 years of temperature and precipitation measurements (*18*). Elevation data, measured by the Shuttle Radar Topography Mission, were reformatted to the same resolution as the other bioclimatic variables and downloaded from WorldClim. Normalized Difference Vegetation Index (NDVI) data were obtained from National Aeronautics and Space Administration's Level 1 and Atmosphere Archive Distribution System website (http://ladsweb.nascom.nasa.gov). The NDVI product used was 1-km monthly data collected from the Terra satellite (product no. MOD13A3). NDVI is a measure of the amount of healthy green vegetation on the ground based on the amount of red and near-infrared reflectance measured by satellite. Low NDVI values are associated with water, snow, sand, rock, and dead vegetation, whereas high values represent healthy green vegetation such as forests and grasslands.

### Ecologic Niche Modeling

An ecologic niche model of Afghanistan was produced in MaxEnt 3.3.3k (http://www.cs.princeton.edu/~schapire/maxent/) (*19*) by using the bioclimatic variables, elevation data, and all locations where wounds were contaminated with mold. The model was projected onto Iraq to determine environmentally similar locations where fungal infections might have been expected in wounds during the Gulf War. MaxEnt, which uses a maximum entropy algorithm, uses

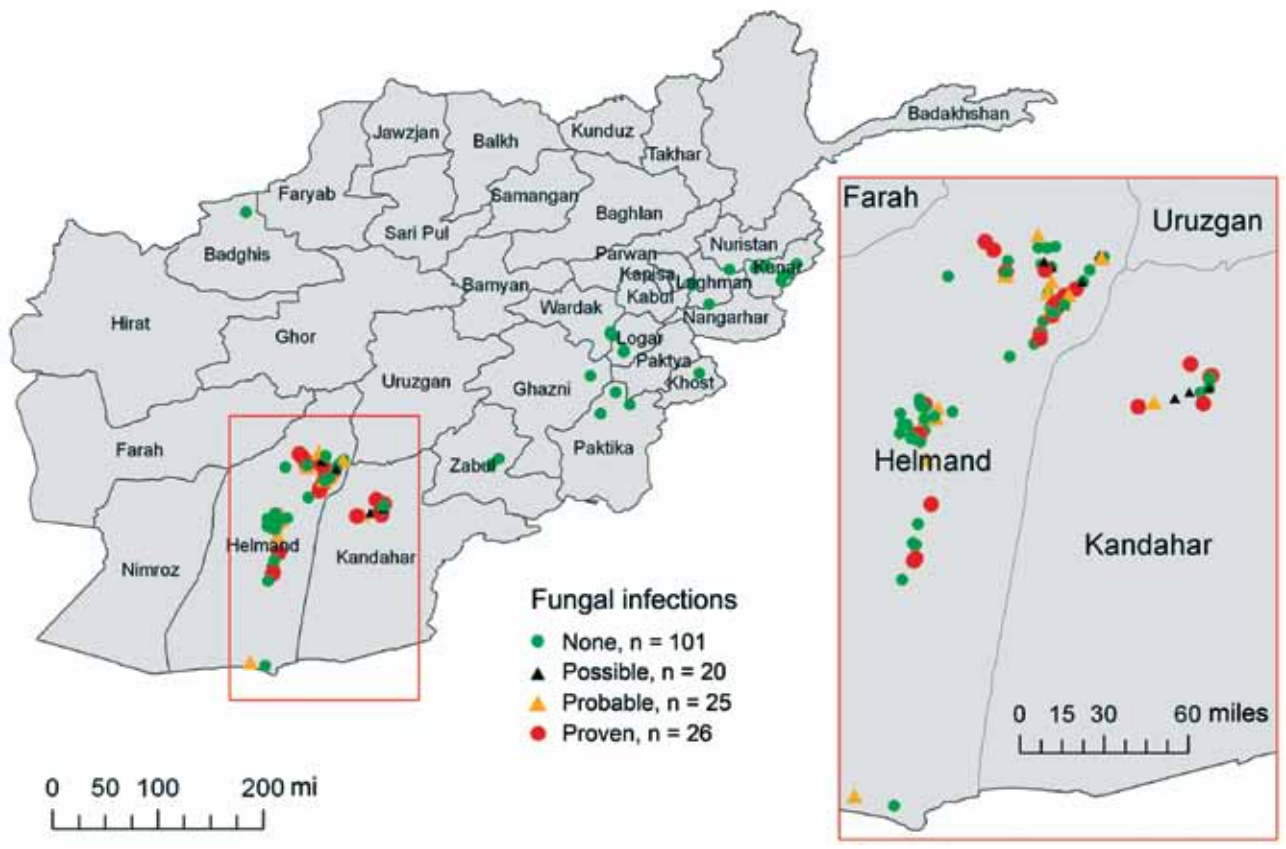


**Figure 1**. Geographic distribution of 71 case-patients with invasive fungal wound infections and 101 matched control-patients. Afghanistan, 2009–2011. Inset shows a detailed view of southern Afghanistan region where most cases originated. The IFI case-patients are classified according to established definitions (*13*). A proven IFI is confirmed by angioinvasive fungal elements on histopathologic examination. A probable IFI had fungal elements identified on histopathologic examination without angioinvasion. A possible IFI had wound tissue grow mold; however, histopathologic features were either negative for fungal elements or a specimen was not sent for evaluation. In addition, to be identified as an IFI, the wound must demonstrate recurrent necrosis after at least 2 surgical débridements. Because injuries frequently occurred in close proximity, some points overlay other points.

a set of raster environmental layers (e.g., temperature and elevation) and information on species presence locations, determines the environmental requirements of the species on the basis of environmental conditions at the presence locations, and produces output maps that predict the probability of presence of a species. MaxEnt uses presence-only data rather than presence/absence data and has been shown to be a high-performing model-building program (*20*) and excels in using small numbers of occurrence points (*21*).

Twenty-five percent of the occurrence points were used for testing the model accuracy (testing points), and the remaining 75% were used for building the model (training points); training and testing points are randomly selected by MaxEnt. As a measure of the accuracy of the model, MaxEnt calculates the area under the curve (AUC) of the receiver operating characteristic of both the training and testing points (*19*,*22*,*23*). To provide estimates of the potential contribution of environmental variables to the model, MaxEnt uses a jackknife test in which the model is run multiple times, with only 1 environmental layer used at a time and then all but 1 variable used, to determine the training gain of each variable in each model (*24*).

### Statistical Analysis

The Wilcoxon rank-sum test was used to compare the geographic characteristics between the 2 regions in Afghanistan. In addition, an unconditional logistic regression model was used to analyze the association between potential environmental risk factors and mold contamination of wounds in a univariable and multivariable analysis. Backward elimination was used to determine which factors that were significant in the univariable analysis remained in the final multivariable model. A correlation analysis was also conducted to examine the relationship between the environmental factors. Statistical analysis was conducted by using SAS version 9.3 (SAS Institute, Inc., Cary, NC, USA). Significance level for all statistical tests was 0.05.

## Results

### Study Population and Injury Characteristics

The location of injury and IFI status of the 172 patients used as the basis for the mold wound contamination analysis are shown in Figure 1. After 167 patients were classified by mold contamination status (101 patients with mold contamination and 66 without), 147 military personnel injured in the southern region of Afghanistan (area favorable to mold wound contamination) and 20 injured in eastern Afghanistan (less frequent mold wound contamination) were included in the analysis (Figure 2). As previously stated, 5 patients (including 1 IFI case-patient) were excluded because the injury occurred outside of the study regions. Cultures from 7 patients in the mold contamination group included in the analysis did not have mold growth, but the patients received a diagnosis of IFIs on the basis of histopathologic examination. Notably, all remaining IFI case-patients sustained injuries in the southern region, whereas both regions contained patients with mold wound contamination.

Although the injury mechanism was predominantly blast (e.g., improvised explosive device, rocket-propelled grenade, and grenade) for military personnel injured in both regions, more service members were injured while dismounted in the southern region of Afghanistan (95% vs. 60%; p<0.001). In addition, patients injured in southern Afghanistan also had significantly higher injury severity scores (median 21 vs. 17.5; p = 0.033) and a more above-knee amputations (46% vs. 5%; p<0.001). Correspondingly, southern Afghanistan also had a significantly higher proportion (50% vs. 5%; p = 0.004) of wounded military personnel that required super-massive transfusions (≥20 units) of packed red blood cell within 24 hours following injury.

### Factors Associated with Mold Wound Contamination

The spatial distribution of patients included in the analysis, based on the presence or absence of mold wound

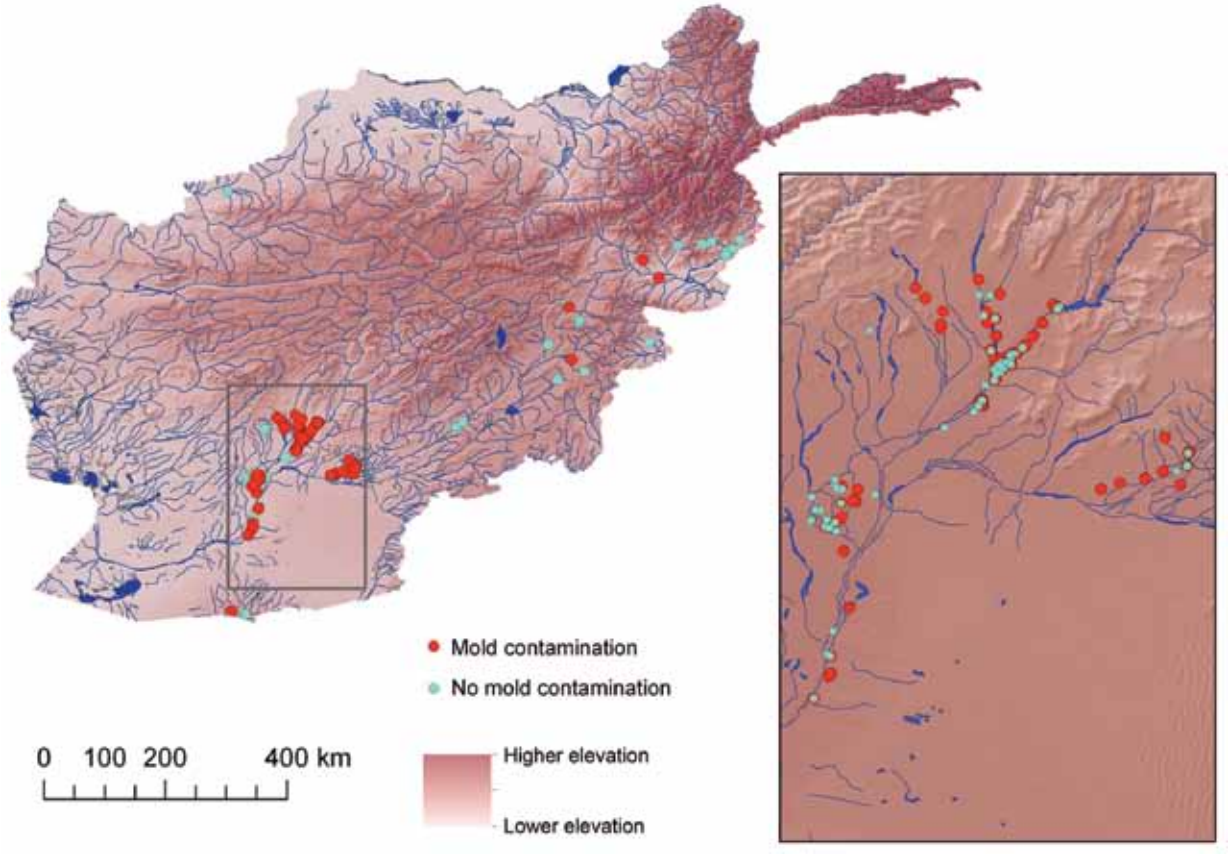


**Figure 2**. Geographic distribution of military personnel with wounds contaminated by mold (n = 101) and control-patients (n = 66), Afghanistan, 2009–2011. Inset shows detail view of southern Afghanistan region where most cases originated. The mold contaminated group includes 7 patients for whom cultures did not show mold growth, but were diagnosed with invasive fungal wound infections (IFIs) on the basis of histopathologic examination. Five patients with injuries sustained outside the study regions were excluded from the analysis (including 1 patient with IFI), but remain visible on the map (i.e., 1 point in western region, 2 in the southernmost point of Helmand Province below the box indicating the southern analysis region, and 2 in Zabul Province between the southern and eastern regions). Because injuries frequently occurred in close proximity, some points overlay other points. The mold contamination points are on top on the overview map and on the bottom in the enlarged inset. Higher elevation is indicated by darker brown.

**Table 1**. Environmental risk factor analysis for mold contamination of wounds, Afghanistan, 2009–2011*

| Environmental factor | Univariable OR (95% CI) | p value† | Multivariable OR (95% CI) | p value‡ |
|---|---|---|---|---|
| NDVI§ | 0.93 (0.49–1.77) | 0.89 | 1.16 (0.58–2.33) | 0.68 |
| Elevation | 1.00 (0.99–1.00) | 0.11 | 1.00 (1.00–1.01) | 0.61 |
| Annual precipitation¶ | 0.99 (0.99–1.00) | 0.01 | – | – |
| Annual mean temperature | 1.09 (0.95–1.24) | 0.22 | 0.84 (0.42–1.67) | 0.61 |
| Temperature annual range | 1.19 (0.97–1.47) | 0.10 | 0.74 (0.50–1.09) | 0.12 |
| Regional area | | | | |
| East | Reference | | Reference | |
| South | 7.76 (2.46–24.4) | 0.001 | 129.9 (7.71–>999)# | 0.001 |

*OR, odds ratio; NDVI, normalized difference vegetation index.
†For univariable analysis.
‡For multivariable analysis.
§The NDVI uses a mathematical formula to quantify the density of healthy green vegetation in a region (*25,26*). The NDVI values were normalized by taking the natural log of the index.
¶Because of its high correlation with the temperature variables, annual precipitation was not included in the final multivariable model.
#The wide CI is due to correlation between the regional and the environmental variables.

contamination, is displayed in Figure 2. Overall, mold was recovered more frequently from wounds of patients injured in southern Afghanistan (61% vs. 20%; p<0.001). When we included patients who did not have mold growth but did have IFIs that were diagnosed on the basis of histopathologic features, 66% of those injured in southern Afghanistan had mold contamination. The variables of region (southern vs. eastern), NDVI, elevation, annual precipitation, annual mean temperature, and annual range of temperature were examined for association with wound mold contamination on a per individual basis (Table 1). Annual precipitation was significantly associated with mold contamination (p = 0.01) on univariable analysis but was not included in the final multivariable model because of its high correlation with temperature variables. Injuries sustained in southern Afghanistan were significantly more likely to be contaminated with mold (p = 0.001), and region was the only factor retained as a statistically significant independent predictor in the final multivariable model (p = 0.001).

**Table 2.** Environmental characteristics of 2 regions in Afghanistan associated with combat-related trauma, June 2009–August 2011*

| Characteristic | Southern Afghanistan, median (IQR) | Eastern Afghanistan, median (IQR) | p value† |
|---|---|---|---|
| NDVI‡ | 7.64 (7.23–7.95) | 7.69 (7.52–8.00) | 0.384 |
| Elevation, m | 902 (859–946) | 1,670 (1,050–2,117) | <0.001 |
| Isothermality§ | 4.2 (4.1–4.2) | 3.4 (3.4–3.5) | <0.001 |
| Temperature, °C | | | |
| Annual mean | 18.8 (18.6–19.5) | 13.9 (10.3–18.3) | <0.001 |
| Seasonality¶ | 880.7 (877.9–884) | 861.7 (853.6–964.0) | 0.471 |
| Mean diurnal range | 17.4 (17.3–17.5) | 13.5 (12.7–15.0) | <0.001 |
| Maximum of warmest month | 40.8 (40.5–41.3) | 33.4 (31.5–36.6) | <0.001 |
| Minimum of coldest month | −0.6 (−0.8 to 0) | −3.8 (−12 to 0.5) | 0.194 |
| Annual range | 41.4 (41.3–41.5) | 36.5 (35.8–43.3) | 0.138 |
| Mean of wettest quarter | 9.6 (9.2–10.3) | 8.1 (4.8–12.5) | 0.681 |
| Mean of driest quarter | 30.1 (29.6–30.8) | 19.3 (17.8–21.3) | <0.001 |
| Mean of warmest quarter | 30.2 (29.9–30.8) | 25.3 (22.5–29.3) | <0.001 |
| Mean of coldest quarter | 7.3 (7.0–7.9) | 2.7 (−2.9 to 7.1) | <0.001 |
| Precipitation, mL | | | |
| Annual | 145 (130–154) | 443 (2,795–546) | <0.001 |
| Wettest month | 39 (35–40) | 89 (64–123) | <0.001 |
| Driest month | 0 | 6 (0–12.5) | <0.001 |
| Seasonality# | 110 (108–115) | 82 (73–90) | <0.001 |
| Wettest quarter | 103 (92–109) | 210 (168–307) | <0.001 |
| Driest quarter | 0 | 28 (8–51) | <0.001 |
| Warmest quarter | 0 | 46 (10–66) | <0.001 |
| Coldest quarter | 90 (80–95) | 125 (113–151) | <0.001 |

*Environmental characteristics were obtained in relation to grid coordinates on a per-individual basis: southern Afghanistan region (147 patients) and eastern Afghanistan region (20 patients). IQR, interquartile range; NDVI, normalized difference vegetation index.
†p values were calculated using Wilcoxon rank-sum test. The regional variables were treated as dependent, whereas the environmental characteristic was independent.
‡NDVI uses a mathematical formula to quantify the density of healthy green vegetation in a region. Higher values indicate healthy green vegetation (e.g., forests), while lower values are associated with water, snow, sand, rock, and dead vegetation (*25,26*). The NDVI values were normalized by taking the natural log of the index.
§Mean diurnal range divided by temperature annual range and then multiplied by 100.
¶SD of weekly mean temperature multiplied by 100.
#Coefficient of variation.

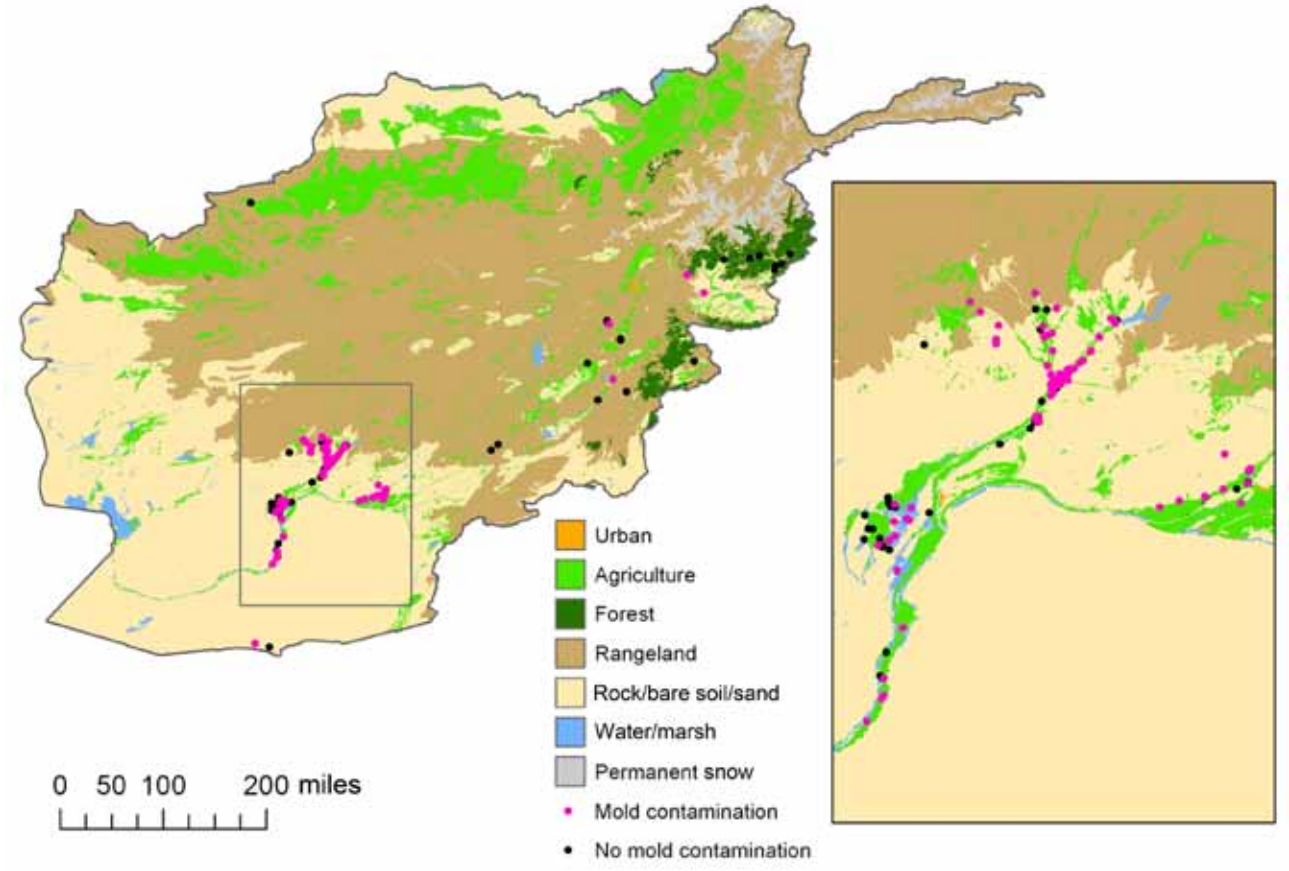


**Figure 3.** Geographic features of southern and eastern Afghanistan study zones, 2009–2011. Inset shows detail view of southern Afghanistan region where most cases originated. Because injuries frequently occurred in close proximity, some points overlay other points. The mold contamination points are on top.

### Regional Environmental Characteristics

Because mold-contaminated wounds were more frequent in southern Afghanistan than in eastern Afghanistan (Figure 2), we compared the environmental factors. The elevation of the southern Afghanistan study zone was significantly lower ($p = 0.001$) than that of the eastern region (Table 2). The regions also exhibited differences in temperature. Southern Afghanistan was generally warmer with the annual mean temperature ($p<0.001$), mean diurnal range ($p<0.001$), and isothermality ($p<0.001$) higher than the eastern region. We found no statistically significant difference in temperature seasonality, as expressed by the SD of the weekly mean temperatures and the annual temperature range between the 2 regions. Although southern Afghanistan had a lower amount of annual precipitation ($p<0.001$) than the eastern region, the variable did not differentiate between rain and snow. Vegetative cover, as categorized by the NDVI, was comparable between the 2 regions.

The southern Afghanistan study zone included the provinces of Kandahar and Helmand. In general, the region is arid and characterized by flat grasslands or rangelands with areas of agriculture. A portion of control-patients sustained injuries in the agricultural areas of southern Afghanistan, but most military personnel injured in this region had mold-contaminated wounds (Figures 2, 3).

The eastern Afghanistan study zone included 9 provinces northeast of the southern study zone. The terrain in eastern Afghanistan is largely mountainous with small forested and agricultural areas. As with southern Afghanistan, the 4 patients with mold-contaminated wounds sustained injuries in the vicinity of agricultural zones (Figures 2, 3). It is notable that the species of molds that grew in patients injured in southern Afghanistan are known to be pathogenic, such as the order Mucorales and *Aspergillus* spp. (Figure 4).

### Ecologic Niche Modeling

A total of 61 points were used to build the model (training points), and 20 points were withheld to test the Afghanistan model. The Afghanistan model had a high AUC value (0.963), indicating that the model is predicting better than a random model (0.5) and in the very high (>0.9) accuracy range (*22*). In the jackknife test, variables of mean

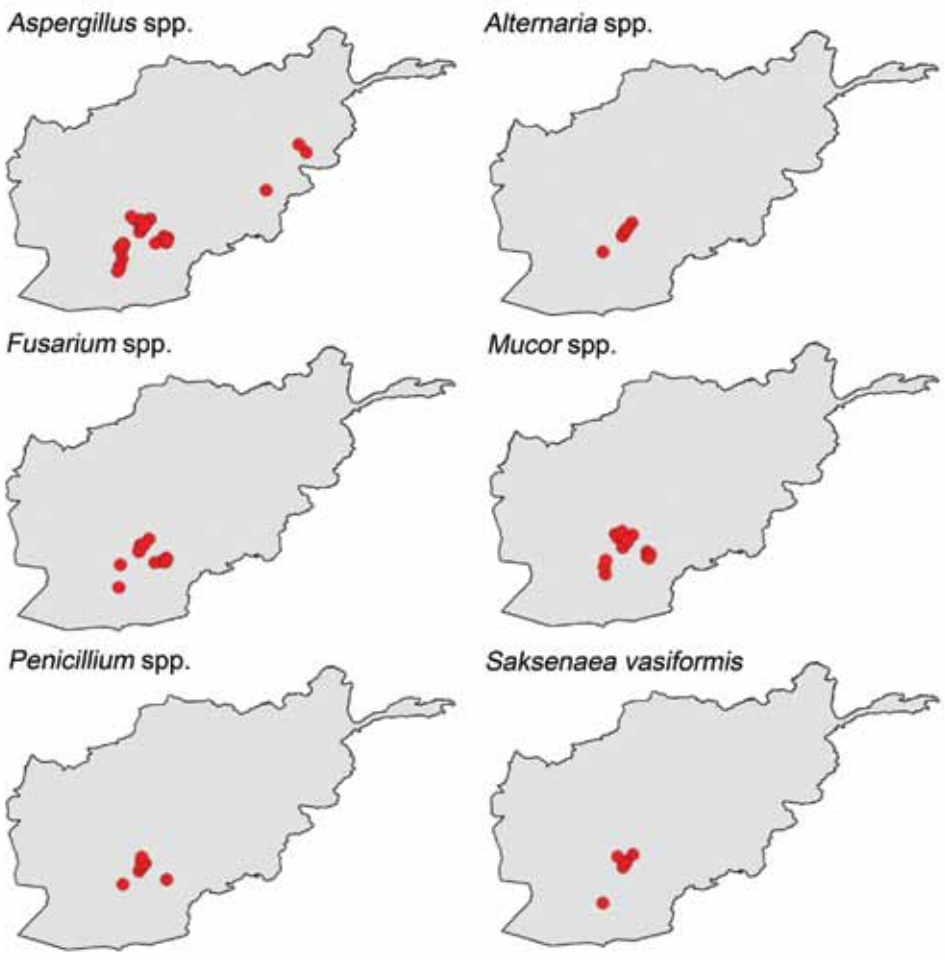


**Figure 4**. Geographic distribution of specific molds isolated from wounds sustained by military personnel in Afghanistan, 2009–2011.

diurnal range, isothermality, and annual precipitation had the highest training gains, which indicated that they had the greatest predictive abilities.

## Discussion

During the recent military conflicts, combat-related IFIs have largely occurred in military personnel who sustained dismounted blast injuries in southern Afghanistan. Although risk factors related to the severity and circumstances of injury have been analyzed (*14*), the effect on disease of the region's climate and environmental conditions has not been previously considered. We compared environmental data from a region in Afghanistan associated with a high risk of mold contamination of wounds (mold was recovered from 61% of the high-risk patients in this cohort) to a region where mold was infrequently reported (20%) among combat wounds. Results of the multivariable analysis confirmed that injuries (on an individual basis) sustained in the southern region of Afghanistan were more likely to be contaminated with mold (odds ratio [OR] 129.9, 95% CI 7.71 to >999), which corroborates clinical observations. Because case-patients with mold contamination were grouped in a similar manner to the IFI case-patients (Figures 1, 2), we believe that these data will be applicable to potential future IFI outbreaks related to combat situations.

Along with the environmental characteristics of a region, circumstances of injury play a major role in creating the potential for mold contamination of wounds and subsequent likelihood of progression to an IFI. Notably, the proportion of military personnel in southern Afghanistan with IFI risk factors (i.e., dismounted blast injuries, massive blood transfusions, and above-knee amputations) was significantly greater than that of personnel in the eastern region. In particular, more personnel in southern Afghanistan were dismounted at the time of injury, sustained a greater number of traumatic above-the-knee amputations, and required more super-massive packed red blood cell transfusions.

In addition, our data indicated that the southern region of Afghanistan was generally warmer than the eastern region with greater isothermality, which is consistent with conditions favorable for mold growth. In particular, high humidity and temperatures of ≈27°C have been reported as

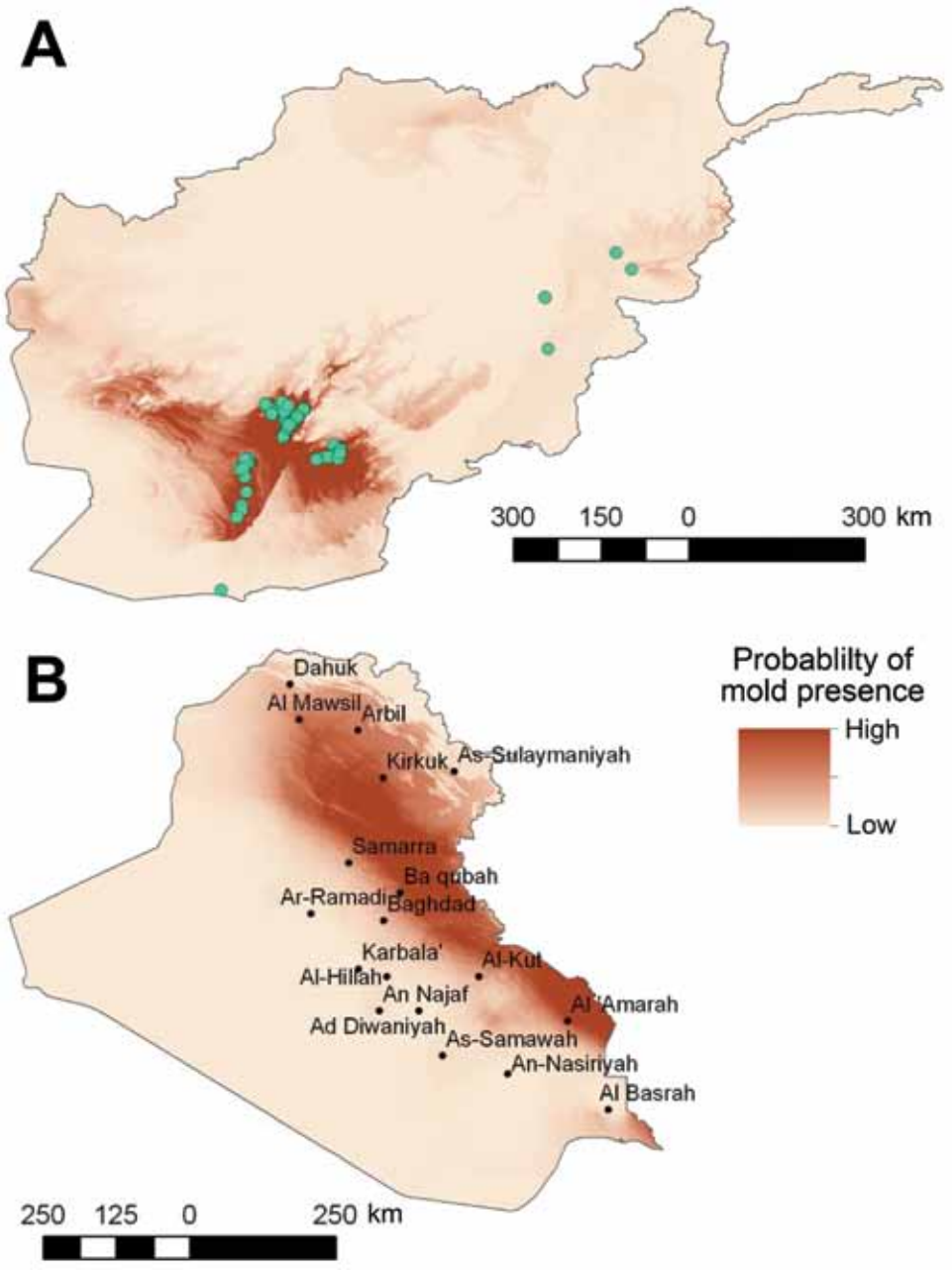


**Figure 5**. Results of ecologic niche modeling in Afghanistan, 2009–2011 (A), and projection of findings onto Iraq (B). Darker red indicates areas estimated to have higher probability of mold presence based on the environmental conditions of mold contamination locations in Afghanistan (green circles).

the optimal conditions for growth of mold from the order Mucorales i (*27*). Similar observations have been reported for *Aspergillus* spp., with optimal temperatures ranging from 25°C to 40°C (*27*). Although precipitation was not retained in the multivariable analysis, it was significantly associated with mold contamination in the univariable analysis (Table 1). Nonetheless, annual precipitation was significantly lower in southern Afghanistan, an arid environment (Table 2). Because moisture is necessary for mold growth, we believe that rivers and agricultural irrigation are playing a large role in southern Afghanistan. Similar NDVI rates in the southern and eastern regions, in spite of differences in annual precipitation rates, lend support to this idea. Another explanation is that eastern Afghanistan has a significantly higher elevation and lower temperatures. Thus, a proportion of the precipitation may have been snow, which is not conducive to mold growth. Future attempts might improve the model by incorporating additional data reflecting ground moisture, runoff, and/or irrigation presence.

Approximately 3,300 species of soil fungi have been identified worldwide (*27*). Analyses of the military personnel with IFIs found that the predominant fungi involved were Mucorales and *Aspergillus* spp. (*6*,*13*). Species in the

order Mucorales are commonly found on decaying organic matter, crop debris, compost piles, animal excreta, and agricultural/forest soils (*27*). *Aspergillus* spp. are another fungi frequently found worldwide in agricultural, forest, grassland, wetland, and desert soils (*28*). Although fungal species are found worldwide, local variations in the incidence of the individual species have been reported in soils and plants due to differences in such factors as temperature, humidity, and host plant species (*29–33*). Cereal crops (e.g., wheat, rice, maize, and barley), which are frequently associated with growth of Mucorales and *Aspergillus* spp. in the soil (*28*,*34*), are predominant in Helmand Province, but production is often affected by resource variability due to drought and flooding. Moreover, agriculture in Afghanistan also includes the illegal growth of poppies for opium production (*35*), with a reported 806 square miles planted across Afghanistan in 2013 (*36*). Notably, provinces in southern Afghanistan (i.e., Helmand and Kandahar) contribute 73% to the overall growth of poppies in Afghanistan (*35*). Agricultural changes such as these in provinces where military personnel are injured may result in differing exposures to soil molds and may explain the geographic variation of the case-patients.

Despite statistically significant differences in the environmental characteristics of the 2 regions, we are not able to definitively conclude that the environmental conditions were directly associated with increased risk for mold wound contamination among wounded military personnel. Nevertheless, one can reasonably assume that the environmental conditions made mold wound growth more likely in conjunction with the specific scenario of mechanism and pattern of injury. Thus, environmental data obtained in our analysis may be extrapolated and used in niche modeling in an effort to speculate on the likelihood of IFIs in different regions with similar circumstances of combat-related injuries. As a theoretical example, we developed an ecologic niche model for Afghanistan (Figure 5) using MaxEnt (*19*) and projected the model on Iraq. The Iraq model shows regions where the environmental conditions are similar to the environmental conditions in southern Afghanistan where mold wound contamination frequently occurred. A very low incidence of combat-related IFI was reported among combat casualties in Iraq (*12*), possibly due to less frequent dismounted blast injuries, However, we did not have the grid coordinates where injuries were sustained to evaluate whether the locations were consistent with our predictive map. Although we cannot draw conclusions about where personnel were injured or how accurate the model is for Iraq, military operations did occur in the areas indicated by the predictive map (Figure 5).

A potential limitation of our analysis is the lack of environmental mold sampling; however, whether this would be contributory or as valuable as wound contamination sampling is not clear. From a biogeographic perspective, sampling wounds for mold contamination is not the ideal sampling method for determining where mold is abundant in the environment. For this study, we were unable to obtain soil and plant samples to confirm or disprove the idea that certain regions are more likely to support the development of IFIs after traumatic injury. Although impractical in a war zone and expensive to implement, given adequate mold samples over a wide geographic region and range of environmental factors, the ecologic niche model developed in this article could be improved and may ultimately be a useful tool. For example, mold samples from soils and vegetation have proven useful in agricultural research in determining factors associated with poor crop health (*30–32*). Thus, similar sampling may prove useful in predicting geographic areas with a higher likelihood for mold wound contamination, allowing clinicians to have a heightened awareness of the risk for IFI among patients with severe traumatic injuries. This awareness will contribute to earlier diagnosis and more timely treatment of the disease.

Overall, our data indicate that the environmental conditions in southern Afghanistan were favorable to mold growth, particularly when a specific mechanism and pattern of injury occurred. We also believe that the specific environmental characteristics may be applied to predictive modeling that would be useful during future military conflicts in situations in which the injury mechanism and pattern match known IFI risk factors.

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# *Haemaphysalis longicornis* Ticks as Reservoir and Vector of Severe Fever with Thrombocytopenia Syndrome Virus in China

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Severe fever with thrombocytopenia syndrome (SFTS) is an emerging hemorrhagic fever in East Asia caused by SFTS virus (SFTSV), a newly discovered phlebovirus. The *Haemaphysalis longicornis* tick has been suspected to be the vector of SFTSV. To determine whether SFTSV can be transmitted among ticks, from ticks to animals, and from animals to ticks, we conducted transmission studies between developmental stages of *H. longicornis* ticks and between ticks and mice. Using reverse transcription PCR, we also analyzed the prevalence of SFTSV infection among *H. longicornis* ticks collected from vegetation in Shandong Province, China. Our results showed a low prevalence of SFTSV among collected ticks (0.2%, 8/3,300 ticks), and we showed that ticks fed on SFTSV-infected mice could acquire the virus and transstadially and transovarially transmit it to other developmental stages of ticks. Furthermore, SFTSV-infected ticks could transmit the virus to mice during feeding. Our findings indicate ticks could serve as a vector and reservoir of SFTSV.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging hemorrhagic fever caused by SFTS virus (SFTSV), a newly discovered phlebovirus in the family *Bunyaviridae* (*1*,*2*). SFTS was reported in China in 2009 (*1*) and subsequently in Korea and Japan (*3*,*4*). Approximately 1,000 SFTS cases are reported each year in China (*5*), where the case-fatality rate is 6.3%–12.0% (*1*,*5*). SFTS cases occur in rural areas of China, where there are shrubs, grasslands, or both, and a high density of *Haemaphysalis longicornis* ticks (*1*,*6*–*9*). Since the first discovery of SFTSV, transmission of the virus by ticks, especially the *H. longicornis* tick, has been proposed (*1*). However, it has not been determined whether SFTSV can be transmitted transstadially and transovarially in ticks or whether ticks can transmit SFTSV to animals. To determine the possibility of such transmission and to determine whether ticks might be a reservoir for SFTSV, we investigated ticks as a possible vector by determining tick-borne transmission of SFTSV during the tick developmental stages and transmission of SFTSV to animals.

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## Materials and Methods

### Collection of Ticks from Vegetation

During June and July 2013, we collected ticks (questing larvae, nymphs, and adults) in Jiaonan County, Shandong Province, China (119°30′–120°30′, 35°35′–36°08′), by flagging over vegetation with a 1-m$^2$ flannel flag. Tick collection was performed for 4 days during the first week of each month between 10:00 AM and 12:00 PM and between 2:00 PM and 5:00 PM; different sites were used for each collection. The ticks were frozen at –80°C until use. Tick species and developmental stages were identified morphologically, and the tick species was molecularly confirmed. The use of the animals and the collection of samples were approved by the bioethics committee of School of Public Health, Shandong University.

### Tick Nucleotide Extraction and Identification by PCR

Ticks were grouped according to their developmental stages; each group contained 40 larvae, 20 nymphs, or 5 adult ticks. Sex of the ticks was ignored. Ticks in each group were homogenized by using metal beads and Buffer RLT in a TissueLyser LT (both from QIAGEN, Hilden, Germany). The total nucleotides (DNA and RNA) were extracted simultaneously by using the AllPrep DNA/RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. Two larval ticks, 1 nymphal tick, and 2 adult ticks were processed individually for rRNA extraction and PCR amplification of the tick mitochondrial 16S rRNA gene. The tick mitochondrial 16S rRNA gene forward

primer (5′-AGTATTTTGACTATACAAAGGTATTG-3′) and reverse primer (5′-GTAGGATTTTAAAAGTTGAACAAACTT-3′) were designed in this study by using a previously published sequence (GenBank accession no. KC203361) as template. The PCR cycle consisted of an initial DNA denaturation step at 95°C for 2 min; 35 cycles of 30 s at 92°C, 30 s at 55°C, and 45 s at 72°C; and a final extension step of 10 min at 72°C. The PCR product was 402 bp and was sequenced on both strands.

### Detection of SFTSV in Ticks by Reverse Transcription PCR (RT-PCR)

The extracted SFTSV RNA was amplified by using a one-step RT-PCR (Access RT-PCR System Kit, Promega, Madison, WI, USA) with primers derived from the small RNA segment of the virus. The RT-PCR primers (CAGCCAGTTTACCCGAACAT and GAAAGACGCAAAGGAGT) and PCR protocol were described previously (*10*). The one-step RT-PCR consisted of 45 min at 45°C and 2 min at 94°C; 40 cycles of 30 s at 94°C, 1 min at 60°C, and 2 min at 68°C; and a final extension of 10 min at 68°C. The RT-PCR product was used as template for a nested PCR, which consisted of a denaturing cycle of 5 min at 95°C; 30 cycles of 20 s at 94°C, 30 s at 56°C, and 1 min at 72°C; and a final extension of 10 min at 72°C. A negative control with sterilized distilled water was run simultaneously. Nested PCR was performed with primers (5′-TGGCTCCGCGCATCTTCACA-3′ and 5′-AGAGTGGTCCAGGATTGCTGTGG-3′), using the RT-PCR product as template (*11*). The amplified DNA was subjected to electrophoresis on a 0.8% agarose gel and visualized under ultraviolet light. The desired 560-bp DNA band was excised and purified from the gel by using the Gel Extraction Kit (QIAGEN). The purified PCR product was cloned into a pMD 19-T vector (TaKaRa Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Positive clones were sequenced on both strands.

### Phylogenetic Analysis

The SFTSV sequences and tick mitochondrial 16S rRNA gene sequences derived from tick nucleotides were compared with sequences in GenBank by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic trees were constructed by using the neighbor-joining method in MEGA5 (*12*), and the robustness of the trees was tested by using 1,000 bootstrap replications. The sequences generated in this study were deposited in GenBank (accession nos. KP300821, KR259990–KR25993 for ticks and KP197680–KP197687 for SFTSV).

### Establishment of a Tick Colony and Transmission Feeding Experiments

A colony of parthenogenic *H. longicornis* tick was established in our laboratory. Initially, adult female ticks collected from vegetation were placed inside a bag attached to a rabbit ear to feed. Each engorged tick was allowed to lay eggs in a plastic tube. Approximately a half clutch of eggs from each female tick was used for RNA extraction and testing for SFTSV by RT-PCR. Larvae were allowed to hatch from the remaining half clutches, and larvae from a single tick that was negative for SFTSV by RT-PCR were used for all subsequent experiments.

SFTSV acquisition and transmission feeding were performed for all stages of ticks by using Kunming mice (Shandong University Experimental Animal Center, Jinan City, China). For the experiments, ticks were placed in feeding capsules, which were prepared from the top of 1-mL screwcap cryovials. The top part of the tube beneath the cap was cut, and the cap of the tube was punctured by using a 26-gauge needle for adequate air exchange. Mice were anesthetized by intraperitoneal injection of 0.1 mL of 10% chloral hydrate. Hair on the back of each mouse was trimmed close to the skin by using a small electric razor, and a capsule was affixed to the back with Kamar glue (Kamar Products Inc., Zionsville, IN, USA). To secure the capsule in place, a round patch of cloth (≈1 cm diameter) with a hole in the middle that was slightly smaller than the capsule (to allow access to the capsule) was placed over the capsule and affixed to the skin with glue. A collar made from thin plastic was placed around the body of the mouse in front of the capsule to prevent the mouse from removing the capsule during grooming.

A total of 50 larvae, 15 nymphs, or 1 adult tick was placed in the capsule on each mouse 24 h after the capsule was affixed to the mouse. Ticks in the capsule were observed daily and collected after completion of feeding. For the virus acquisition feeding, each mouse was inoculated intraperitoneally with DH82 cells containing $10^6$ plaque forming units of SFTSV, and ticks were fed on the SFTSV-infected mice for 3–8 days until engorged. For virus transmission feeding, SFTSV-infected ticks were fed on non-infected mice for 3–8 days until engorged. We collected blood samples from each mouse 7, 14, and 21 days after tick feeding for detection of SFTSV. The SFTSV in the laboratory-reared ticks and in the mouse blood was detected by RT-PCR using the primer pair CAGCCACTTCACCCGAACAT and AAGGAAAGACGCAAAGGAG, which was designed in this study. The amplification cycles were the same as those described above for the RT-PCR for detection of SFTSV in ticks from vegetation. The PCR product was 560 bp.

### Detection of SFTSV RNA and Antibody in Blood of Mice after Tick Feeding

Mouse blood samples were collected weekly for 3 weeks. Total RNA was extracted from each blood sample by using the RNeasy Mini Kit (QIAGEN) and was used as a template

for amplification (Access RT-PCR System; Promega) of SFTSV. Primers for RT-PCR and the PCR protocol were as described in the preceding paragraph. SFTSV antibody was assayed by using SFTSV-infected DH82 cells as antigens. The cells were cultivated in a 96-well plate, fixed with 4% paraformaldehyde, and used as antigens for immunofluorescence assays (IFAs) to detect SFTSV antibodies in serum samples from mice fed on by SFTSV-infected ticks.

## Results

### The Prevalence of SFTSV in Ticks from Vegetation

We collected 3,300 ticks from vegetation, morphologically identified them as *H. longicornis* ticks, and molecularly confirmed the identification by sequencing the mitochondrial 16S RNA gene of representatives of larval, nymphal, and adult ticks (Figure 1). To detect SFTSV, we pooled the ticks according to their developmental stage, and tested each pool for SFTSV RNA by RT-PCR and nested PCR. The prevalence of SFTSV infection in each stage of the tick was determined by the assumption that a positive pool of ticks contained 1 SFTSV-infected tick. The prevalence of SFTSV infection was 0.2% (8/3,300) among ticks of all stages. The prevalence of SFTSV infection was 0 among 120 larvae, 0.1% (2/1,620) among nymphs, and 0.4% (6/1,560) among adult ticks. Phylogenetic analysis showed that all sequences of SFTSV amplified from ticks were clustered with SFTSV from Shandong Province and other places in China (Figure 2). These results suggested that nymph and adult ticks were infected with SFTSV, but the rate of carriage was very low.

### SFTSV Infection of Ticks by Acquisition Feeding and Tick Transovarial Transmission of SFTSV

To determine whether ticks could be infected by SFTSV, we fed all stages (larvae, nymphs, and adults) of *H. longicornis* ticks on SFTSV-infected mice. In larval acquisition feeding, the engorged larvae were tested for SFTSV by RT-PCR before or after molting. Five larval ticks were grouped together as a pool for RNA extraction and RT-PCR amplification of SFTSV. The minimum infection rate (assuming that an infected pool of ticks contained 1 SFTSV-infected tick) was 18% (9/50) for engorged larval pools and 11.7% (14/120) for molted nymphs (Table 1). These results indicated that larvae could acquire SFTSV from infected mice and transstadially transmit SFTSV to nymphs.

In nymphal acquisition feeding, 10 engorged nymphs were tested individually, and all were found to be infected with SFTSV; however, only 20% (2/10) of the adults ticks derived from the nymphs were infected with SFTSV (Table 1). These results indicated that nymphs could acquire SFTSV from infected mice and transstadially transmit SFTSV to adult ticks.

For adult tick acquisition feeding, uninfected adult ticks were fed on SFTSV-infected mice until completion of feeding. Thirteen engorged adult ticks were tested individually for SFTSV, and 6 (46.2%) of them were infected with SFTSV, as determined by RT-PCR. Thirteen

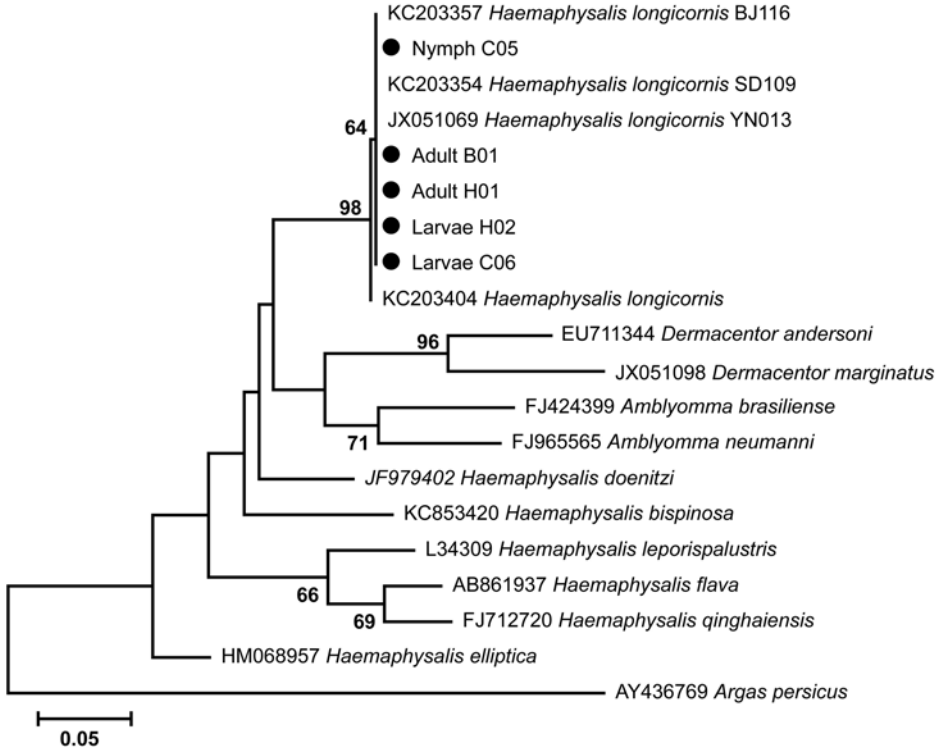


**Figure 1.** Phylogenetic analysis of mitochondrial 16S rRNA gene of ticks collected during June–July 2014 from Jiaonan County, Shandong Province, China. The results showed that the larval, nymphal, and adult ticks (indicated by black dots) were all *Haemaphysalis longicornis* ticks. Scale bar represents nucleotide substitutions per site.

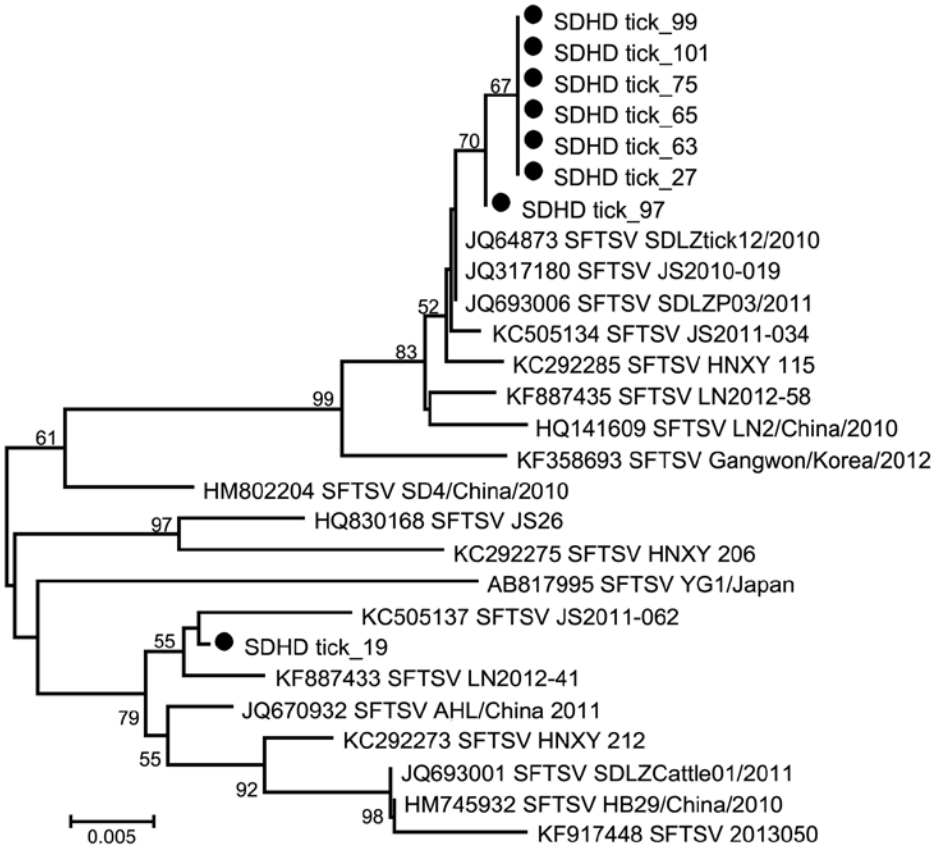


**Figure 2.** Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus (SFTSV) small segment sequences from ticks collected during June–July 2014 from Jiaonan County, Shandong Province, China. Dots indicate SFTSV sequences amplified from ticks in this study; GenBank accession numbers are shown for other sequences. Scale bar represents nucleotide substitutions per site.

engorged adult ticks had oviposited, and the larvae hatched. Larvae derived from each tick were tested as a group for SFTSV RNA by RT-PCR, and 53.8% (7/13) of the larval pools were infected with SFTSV (Table 1). The results of these experiments indicated that adult female ticks could transovarially transmit SFTSV.

### Tick Transmission of SFTSV to Mice

To determine whether SFTSV could be transmitted to mice by tick bite, we fed SFTSV-infected nymphs and adults on Kunming albino mice. For study of nymph feeding transmission of SFTSV, 10 uninfected mice were fed upon by 10 nymphs molted from larvae that fed on SFTSV-infected mice until engorged. At 7, 14, and 21 days after the feeding, blood samples were obtained from the mice and examined by RT-PCR to detect SFTSV; viral RNA was detected in 40% (4/10) of the mice. These results indicated that nymphs transmitted SFTSV to mice through feeding.

For study of adult tick feeding transmission of SFTSV, the adult ticks were molted from engorged nymphs obtained from the 10 mice mentioned above. To determine whether SFTSV was transmitted from nymphs to adult ticks and whether adult ticks could transmit SFTSV to mice, 20 adult ticks were fed individually on 20 naive mice until engorged. At 7, 14, and 21 days after the feeding, blood samples were obtained from the mice and examined by RT-PCR to detect SFTSV; viral RNA was detected in 10.0% (2/20) of the mice on days 7 and 14. These results indicated that the adult ticks used in this study were infected in the larval stage by feeding on SFTSV-infected mice.

Using an IFA, we determined whether SFTSV antibodies were present in serum samples from mice fed upon by nymphal and adult ticks. All SFTSV RT-PCR–positive mouse serum samples were also positive by IFA at various titers (Table 2; Figure 3), but none of the SFTSV RT-PCR–negative mouse samples were positive by IFA. The results indicated that larvae acquired SFTSV from infected mice and transmitted the virus transstadially to nymphs and adult ticks, and nymphs and adult ticks transmitted SFTSV to mice during feeding.

**Table 1.** Rate of SFTSV infection among *Haemaphysalis longicornis* ticks after virus acquisition feeding and molting*

| Tick stage | No. SFTSV-infected ticks after acquisition feeding /no. total (%) | No. ticks with transstadially or transovarially transmitted SFTSV/no. total (%) |
|---|---|---|
| Larval | 9/50 (18.0) | 14/20 (70.0) |
| Nymphal | 10/10 (100) | 2/10 (20.0) |
| Adult | 6/13 (46.2) | 7/13 (53.8) |

*SFTSV, severe fever with thrombocytopenia syndrome virus.

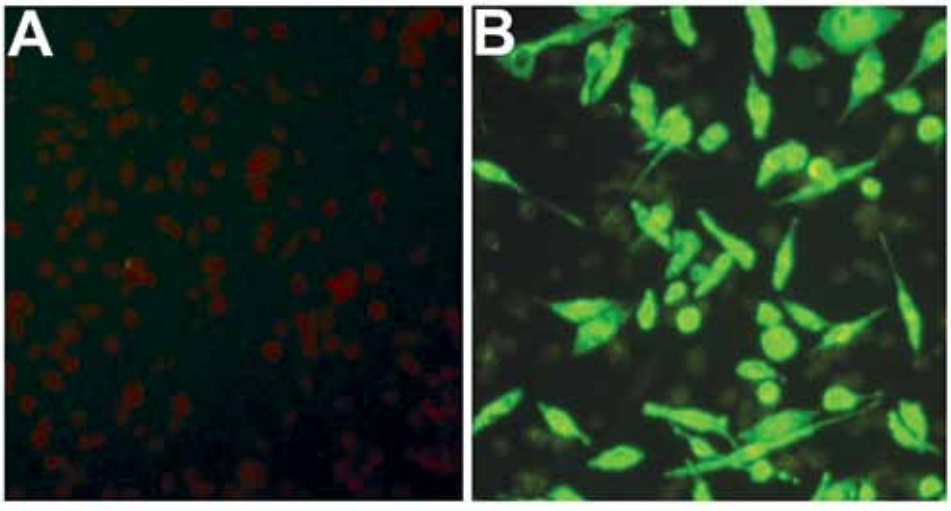


**Figure 3.** Immunofluorescence assay detection of severe fever with thrombocytopenia syndrome virus (SFTSV) antibodies in serum samples from mice fed by SFTSV-infected ticks. A) Normal mouse serum (negative control) reacting with SFTSV-infected DH82 cells; B) infected mouse serum (1:128, mouse no. 4) reacting with SFTSV-infected DH82 cells.

## Discussion

SFTSV has been believed to be a tick-borne virus since it was first discovered because it was detected in ticks collected from animals (*1*). However, detection of a virus in feeding ticks collected from animals does not confirm that the tick is a vector or reservoir of the virus; it is possible that ticks can acquire a virus from the blood of an infected animal but not maintain it transstadially and transmit it during feeding. Several studies have tried to demonstrate that ticks are a vector and reservoir of SFTSV (*13*,*14*). These studies either failed to detect SFTSV in a large quantity of ticks (n = 11,739) (*13*) or demonstrated very low prevalence of SFTSV in ticks collected from vegetation (*14*). A recent study from South Korea demonstrated that SFTSV was detected in all developmental stages of *H. longicornis* ticks, but the prevalence of infection was low in larvae (0.6% [2/350]) and nymphs (0.4% [38/10, 436]) (*14*). In this study, we demonstrated that the prevalence of SFTSV infection in ticks collected from vegetation is also low (0.2%). A recent study reported a slightly higher prevalence of SFTSV infection (2.2%) in *H. longicornis* ticks collected from vegetation from Shandong Province (*15*). The findings from these studies demonstrate that the prevalence of SFTSV infection in tick populations is very low. The reason for the low SFTSV infection rate in nature is not clear. It may be that SFTSV is detrimental to the infected tick and affects its survival. The low SFTSV prevalence in ticks suggests that ticks alone may not be sufficient to maintain SFTSV in nature; circulation of SFTSV between the tick vector and mammalian amplifying hosts may be required for SFTSV maintenance in nature. In previous studies, we and others demonstrated that SFTSV can infect domesticated animals (goats, sheep, cattle, dogs, and chickens) (*6*–*9*) and wild small mammals (mice, rats, and Asian house shrews) (*16*). These animals may be amplifying hosts and ticks may be a vector and reservoir host of SFTSV.

This study and previous studies (*15*,*17*) demonstrated that *H. longicornis* is the predominant tick species in eastern China. *H. longicornis* ticks feed on domesticated and wild animals, including goats, sheep, cattle, pigs, deer, cats, dogs, rats, mice, hedgehogs, chickens, and other birds, as well as on humans (*18*). In rural China, domesticated animals, especially goats and dogs, often roam freely in the environment and maintain a high tick population around farm houses and may increase the risk of SFTSV infection in humans.

One limitation of this study is that a single species of tick was evaluated as a vector and reservoir of SFTSV. The virus has also been detected in *Rhipicephalus microplus* (formerly *Boophilus microplus*), *Amblyomma testudinarium*, and *Ixodes nipponensis* ticks in China and South Korea (*19*,*20*). However, these ticks are not dominant ticks in eastern China, and whether these tick species play a role in the natural cycle of SFTSV needs to be further investigated. SFTSV has also been detected by RT-PCR in *Leptotrombidium scutellare* mites collected from *Apodemus agrarius* mice and from *Laelaps echidnina* mites collected from *A. agrarius* mice and goats in Jiangsu Province, China (*21*), and from the gadfly (species not defined) in the family

**Table 2**. Detection of SFTSV RNA and antibodies in serum samples from mice fed on by SFTSV-infected *Haemaphysalis longicornis* ticks*

| Mouse no. | IFA antibody titer | RT-PCR detection of RNA |
|---|---|---|
| Fed on by nymphal ticks | | |
| 1 | 128 | Positive |
| 2 | 1,024 | Positive |
| 3 | 64 | Positive |
| 4 | 512 | Positive |
| Fed on by adult ticks | | |
| 5 | 64 | Positive |
| 6 | 128 | Positive |

*IFA, indirect immunofluorescence assay; RT-PCR, reverse transcription PCR; SFTSV, severe fever with thrombocytopenia syndrome virus.

*Tabanidae* in Henan Province, China (*17*). Other than ticks, the role of other bloodsucking insects as the vector and reservoir for SFTSV needs to be further investigated.

In this study, we demonstrated that *H. longicorn* ticks at each developmental stage acquired SFTSV during feeding on experimentally infected mice and transmitted SFTSV to mice during feeding. For acquisition feeding, the larval tick appeared to be less efficient than nymphal and adult ticks at acquiring SFTSV from infected mice. However, the difference between infection acquisition for larval ticks and for nymphal and adult ticks may be due to the manner in which we determined the prevalence of SFTSV infection among the different stages of ticks. For larval ticks, prevalence was determined on the basis of the minimum infection rate that was calculated by using pools of ticks; for nymphal and adult ticks, prevalence was determined on the basis of the infection rate that was calculated by using individual ticks. The minimum infection rate may underestimate the true infection rate because a positive pool is counted for only 1 positive tick even though the pool may contain >1 positive tick. We also demonstrated that SFTSV can be transstadially transmitted from larvae to nymphal and adult ticks. However, we may have underestimated the efficiency of transstadial transmission in each tick developmental stage because a larval population fed on an infected mouse was used for transstadial transmission, and the larvae may have included SFTSV-infected and noninfected ticks.

In this study, tick acquisition of SFTSV and transstadial transmission of SFTSV were demonstrated by RT-PCR detection of SFTSV RNA in ticks and confirmed by IFA detection of antibodies to SFTSV in mice fed on by SFTSV-infected nymphal and adult ticks. However, transovarial transmission of SFTSV in ticks was demonstrated only by RT-PCR, which may need further confirmation by isolation of SFTSV from tick eggs or larvae or from mice fed on by hatched larvae or by detection of antibodies to SFTSV in mice fed on by hatched larvae.

In conclusion, all developmental stages of *H. longicorn* ticks can acquire SFTSV by feeding on experimentally infected mice, and the ticks can transmit SFTSV to mice during feeding. The virus can be passed transstadially and transovarially in the developmental stages of the tick. However, the prevalence of SFTSV infection among ticks collected from vegetation is quite low, suggesting that ticks alone (at least *H. longicorn* ticks) may not be sufficient to maintain the virus in nature.

## Acknowledgments

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# Delayed Disease Progression in Cynomolgus Macaques Infected with Ebola Virus Makona Strain

**Andrea Marzi, Friederike Feldmann, Patrick W. Hanley, Dana P. Scott, Stephan Günther, Heinz Feldmann**

In late 2013, the largest documented outbreak of Ebola hemorrhagic fever started in Guinea and has since spread to neighboring countries, resulting in almost 27,000 cases and >11,000 deaths in humans. In March 2014, Ebola virus (EBOV) was identified as the causative agent. This study compares the pathogenesis of a new EBOV strain, Makona, which was isolated in Guinea in 2014 with the prototype strain from the 1976 EBOV outbreak in the former Zaire. Both strains cause lethal disease in cynomolgus macaques with similar pathologic changes and hallmark features of Ebola hemorrhagic fever. However, disease progression was delayed in EBOV-Makona–infected animals, suggesting decreased rather than increased virulence of this most recent EBOV strain.

Ebola virus (EBOV) was discovered 1976 in central Africa and has since then caused multiple localized outbreaks of Ebola hemorrhagic fever (EHF) in humans and great apes in this area (*1*). In December 2013, an EBOV outbreak started in West Africa, in rural eastern Guinea (*2*). From there the disease spread to the neighboring countries of Liberia, Sierra Leone, and Mali and has been imported into Nigeria, Senegal, Spain, the United Kingdom, and the United States (*3*). Almost 27,000 human cases and >11,000 deaths have been documented, making this the largest EBOV outbreak on record (*3*).

Although no approved treatment or vaccine is available for Ebola, this outbreak emphasizes the urgent need for countermeasures. Recently, several experimental EBOV therapeutics and vaccines have been accelerated for licensure and clinical trials are underway (www.clinicaltrials.gov), underlining the severity of the situation for global public health.

The current EHF outbreak was caused by a new strain of EBOV, designated EBOV-Makona, which was first isolated in Guinea in 2014 (*2*). To determine the virulence of the new EBOV-Makona strain from West Africa in the standard macaque animal model, we infected cynomolgus macaques with EBOV-Makona (*2*) and compared infection with that caused by the prototype EBOV strain Mayinga (EBOV-Mayinga) isolated in 1976 (*1*). These virus isolates are 97% identical in their genomic sequence (*2*).

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## Materials and Methods

### Biosafety and Animal Ethics Statements

All infectious work with EBOV was performed by using standard operating procedures approved by the Rocky Mountain Laboratories (RML) Institutional Biosafety Committee in the maximum containment laboratory at the RML, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Hamilton, MT, USA). Animal work was performed in strict accordance with the recommendations described in the Guide for the Care and Use of Laboratory Animals of the National Institute of Health, the Office of Animal Welfare and the United States Department of Agriculture. Procedures were conducted in animals anesthetized with ketamine by trained personnel under the supervision of veterinary staff. All efforts were made to ameliorate the welfare and minimize animal suffering in accordance with the Weatherall report on the use of nonhuman primates in research (https://royalsociety.org/policy/publications/2006/weatherall-report/).

Animals were housed in adjoining individual primate cages that enabled social interactions, under controlled conditions of humidity, temperature, and light (12-h light:12-h dark cycles). Food and water were available ad libitum. Animals were monitored at least twice a day and fed commercial monkey chow, treats, and fruit twice a day by trained personnel. Environmental enrichment consisted of commercial toys. Early endpoint criteria, as specified by the RML Animal Care and Use Committee–approved clinical score parameters, were used to determine when animals should be humanely euthanized.

### Challenge Viruses

EBOV-Mayinga (passage 5) (*1*) and EBOV-Makona C07 (passage 1) (*2*) were used. These 2 viruses were propagated

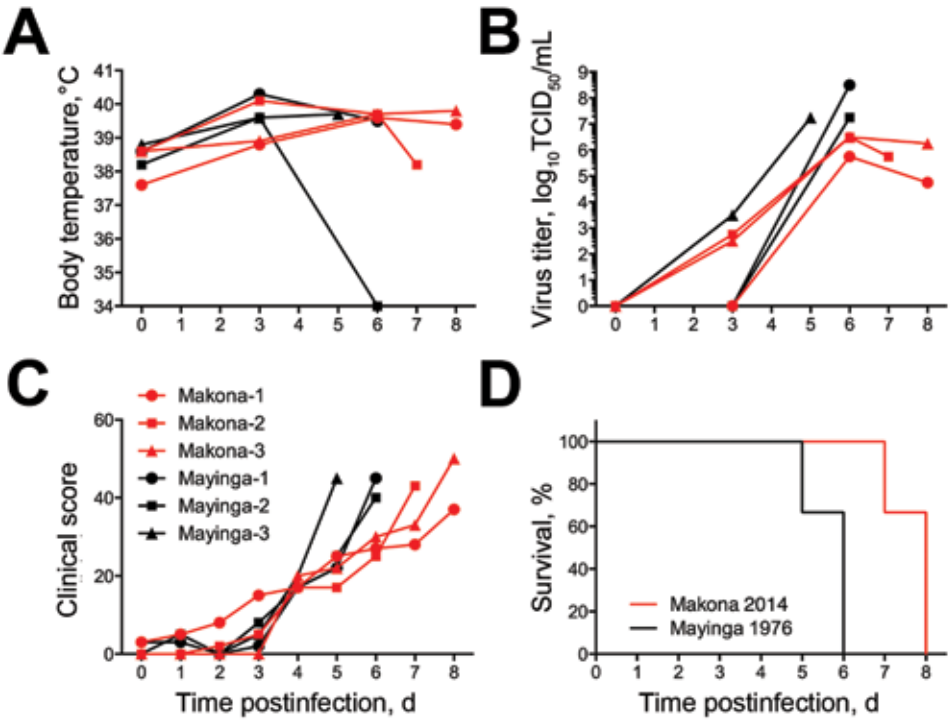


**Figure 1.** Clinical parameters for 6 cynomolgus macaques infected with Ebola virus strains Makona or Mayinga. Parameters were measured for each animal on day of examination and time of euthanasia. A) Temperature profiles. B) Virus titer (viremia). $TCID_{50}$, 50% tissue culture infectious dose. C) Daily clinical scorel. D) Survival curves showing significant difference in time to death between both groups (p = 0.0295, by Mantel-Cox test).

on Vero E6 cells, titered on Vero E6 cells, and stored in liquid nitrogen.

### Study Design

Six cynomolgus macaques (*Macaca fascularis*) (4 male and 2 female animals, age range 6–8 years, weight range 3–7 kg) were used in this study (3 animals/group). All animals were challenged intramuscularly at 2 anatomic locations, the left and right caudal thigh, on day 0 with a dose of 1,000 PFU of EBOV-Mayinga or EBOV-Makona. The animals were observed at least twice a day for clinical signs of disease according to an *Institutional Animal Care and Use Committee*–approved and previously published scoring sheet (*4*) and were humanely euthanized when clinical signs indicated terminal disease on the basis of pre-established endpoints. Blood samples were collected on days 0, 3, and 6 after infection and at time of euthanasia. A full necropsy was performed at the end of the study, and organs were harvested for virologic and pathologic analyses.

### Virus Loads

For determination of virus loads in macaque blood and tissue samples, Vero E6 cells were seeded in 48-well plates the day before titration. Tissues were homogenized in 1 mL plain Dulbecco minimal essential medium, and tissue and blood samples were serial diluted 10-fold. Medium was removed from cells and triplicates were inoculated with each dilution. After 1 h, Dulbecco minimal essential medium supplemented with 2% fetal bovine serum, penicillin/streptomycin and L-glutamine was added and incubated at 37°C. Cells were monitored for cytopathic effect, and 50% tissue culture infectious dose was calculated for each sample by using the method of Reed and Muench (*5*).

### Hematologic and Chemical Analyses

Total leukocyte counts; lymphocyte, platelet, reticulocyte, and erythrocyte counts; hemoglobin levels; hematocrit values; mean cell volumes; mean corpuscular volumes, and mean corpuscular hemoglobin concentrations were determined from blood containing EDTA by using the HemaVet 950FS Analyzer (Drew Scientific, Dallas, TX, USA). Serum biochemical analysis was conducted by using the Piccolo Xpress Chemistry Analyzer and Piccolo General Chemistry 13 Panel Discs (Abaxis, Union City, CA, USA).

### Serum Cytokine Levels

Macaque serum samples were inactivated by using γ-irradiation (5 MRad) and removed from the maximum containment laboratory. Serum samples were then diluted 1:2 in serum matrix for analysis by using the Milliplex Non-Human Primate Magnetic Bead Panel (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. Concentrations of tumor necrosis factor-α, interleukin 6 (IL-6), IL-12/23p40, IL-8, monocyte chemotactic protein 1, IL1Ra, soluble CD40L (sCD40L), IL-15, interferon-g (IFN-g), IL-4, and IL-17 were determined for all samples by using the Bio-Plex 200 System (BioRad Laboratories, Hercules, CA, USA).

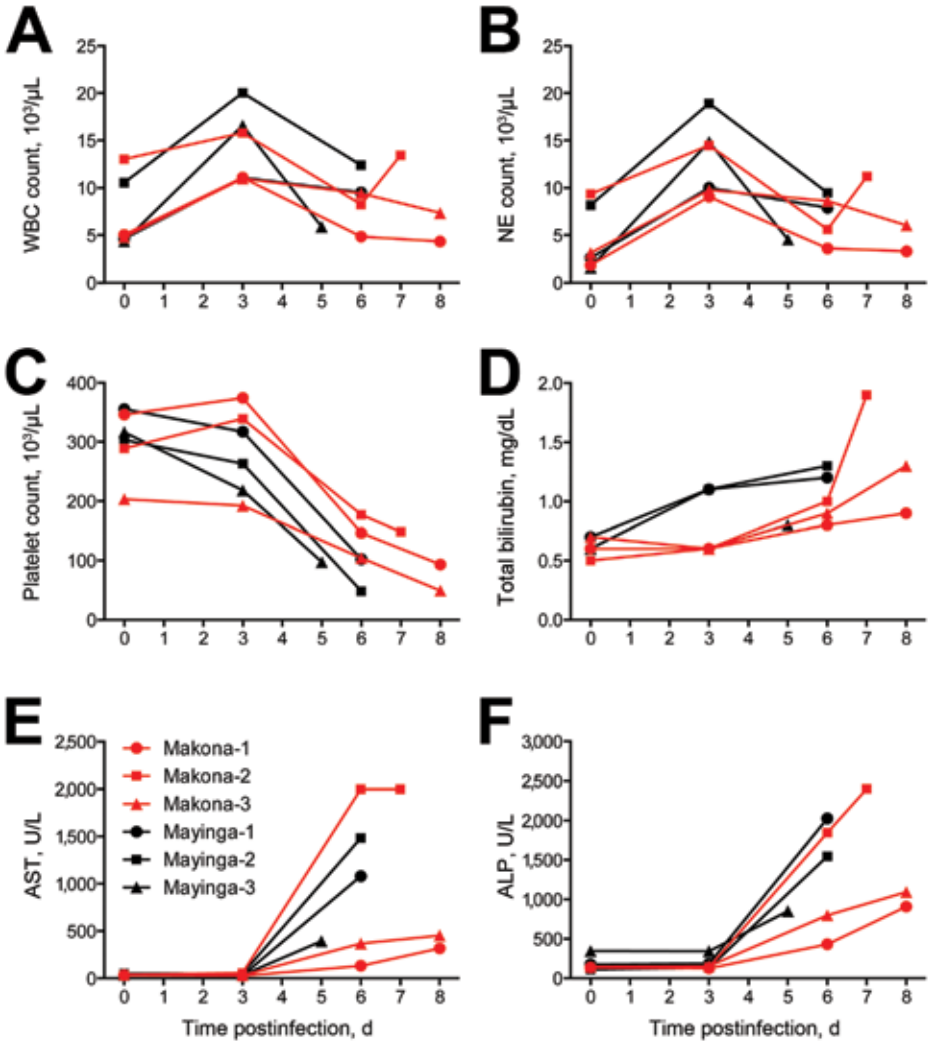


**Figure 2.** Blood and serum parameters for 6 cynomolgus macaques infected with Ebola virus strains Makona or Mayinga. Parameters were measured for each animal on day of examination and time of euthanasia. A) Leukocyte counts. WBC, white blood cell. B) Neutrophil (NE) counts. C) Platelet counts. D) Bilirubin levels. E) Aspartate aminotransferase (AST) levels. F) Akaline phosphatase (ALP) levels.

### Histopathologic and Immunohistochemical Analyses

Analyses were performed with macaque tissues. After fixation/inactivation for 7 days in 10% neutral-buffered formalin, samples were removed from high containment. Subsequently, tissue samples were embedded in paraffin and tissue sections were stained with hematoxylin and eosin. Liver and spleen samples were evaluated in detail, and the following scoring system was applied: 0 = no lesions; 1 = small number of necrotic cells; 2 = moderate necrosis; 3 = significant necrosis; 4 = coalescing necrosis; 5 = diffuse necrosis. To detect EBOV antigen, immunohistochemical analysis was performed by using polyclonal rabbit serum against Ebola virus viral protein 40 as described (*6*).

### Statistical Analyses

A log-rank (Mantel-Cox) test was performed to analyze time to death between the 2 groups. Statistical significance was determined at a level of 0.05. All analyses were conducted by using Prism software (GraphPad Software, San Diego, CA, USA).

## Results

### Disease Progression

Six cynomolgus macaques were randomly divided into 2 groups and infected intramuscularly with 1,000 PFU of EBOV-Makona or EBOV-Mayinga. The targeted challenge dose was confirmed by back-titration of the inoculum on Vero cells. On days 3 and 6 postinfection, all macaques were febrile (temperature >38.5°C) (Figure 1, panel A), and animals showed a decrease in food intake and general activity starting at day 4 postinfection. A prominent, macular, cutaneous rash developed in the 3 EBOV-Mayinga–infected animals throughout their bodies starting on day 4 postinfection. In contrast, this characteristic sign of EHF developed in only 2 of the 3 EBOV-Makona–infected animals on day 6, and the rash was more faint and restricted to the arms, legs, chest, and face.

Unlike in current human EHF cases, diarrhea was only observed temporarily in 1 EBOV-Makona–infected and in none of the EBOV-Mayinga–infected animals. Viremia was detectable as early as day 3 postinfection with no clear distinction among the 2 groups, but end titers were in general higher in EBOV-Mayinga–infected animals (Figure 1, panel B). The 3 EBOV-Mayinga–infected animals had to be euthanized on days 5 and 6 postinfection (1 and 2 animals, respectively) because of severity of disease and after reaching the critical clinical score for euthanasia according to approved animal study protocol (Figure 1, panels C, D) (*4*). In contrast, EBOV-Makona infection resulted in slower disease progression; animals were euthanized on days 7 and 8 postinfection (1 and 2 animals, respectively) (Figure 1, panels C, D), a distinction that was significant ($p = 0.0295$).

### Hematologic and Chemical Analyses

An increase in leukocytes (Figure 2, panel A) was noted in all 6 animals on day 3 postinfection with concurrent neutrophilia (Figure 2, panel B) and lymphopenia, a phenomenon that has been reported in EBOV-infected macaques (*7–9*). Platelet levels decreased over time in all animals, and thrombocytopenia developed during days 3-6 postinfection (Figure 2, panel C). Although the overall hemogram changes were similar for both groups, EBOV-Makona–infected animals often showed a delay and less severe changes than EBOV-Mayinga–infected animals. Animals in both groups showed mild increases in levels of total bilirubin (Figure 2, panel D) and moderate to severe increases in levels of liver enzymes, such as aspartate aminotransferase (Figure 2, panel E) and alkaline phosphatase (Figure 2, panel F). These findings are consistent with those of acute, diffuse hepatic necrosis, which is commonly found with EBOV infection in macaques (*7–9*). Again, increases in liver enzyme levels were delayed in 2 of the 3 EBOV-Makona–infected animals and did not always reach the levels for EBOV-Mayinga–infected animals.

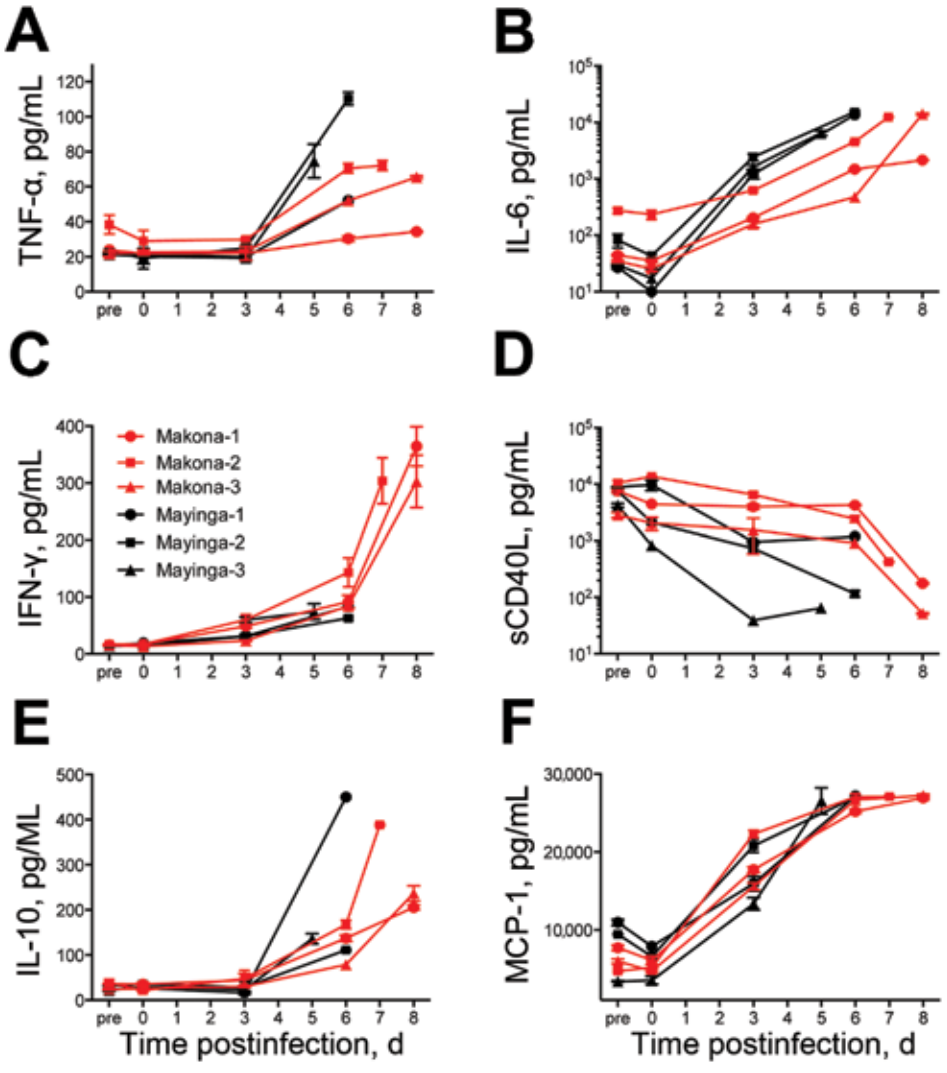


**Figure 3.** Serum cytokine and chemokine levels for 6 cynomolgus macaques infected with Ebola virus strains Makona or Mayinga. A) Tumor necrosis factor-α (TNF-α); B) Interleukin-6 (IL-6); C) Interferon-γ (IFN-γ); D) Soluble CD40 ligand (sCD40L); E) IL-10; and F) Monocyte chemotactic protein 1 (MCP-1). Kinetics were analyzed in serum samples of each animal collected on days of examination and time of euthanasia.

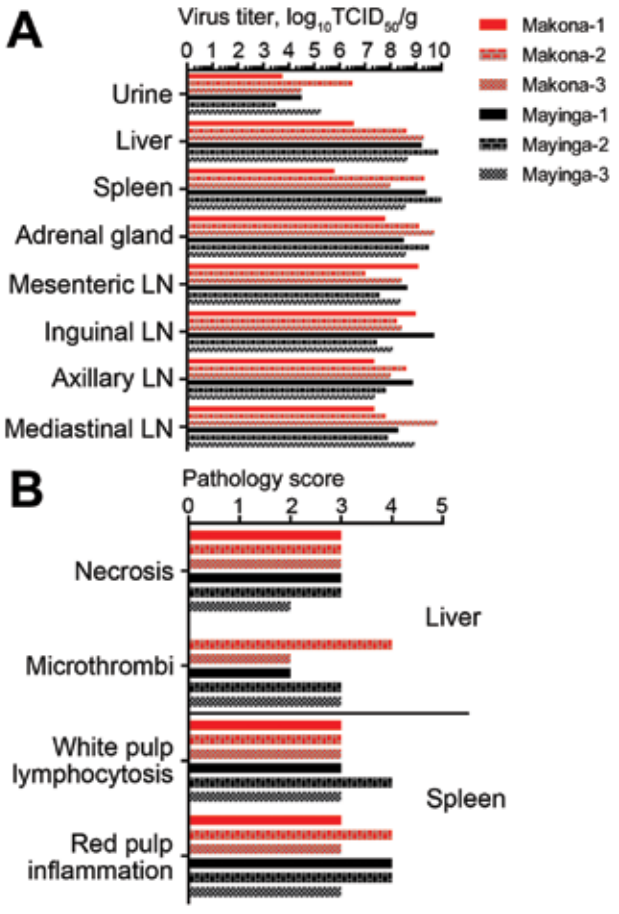


**Figure 4.** Virologic and pathologic results for 6 cynomolgus macaques infected with Ebola virus strains Makona or Mayinga. A) Viral infectivity titers were determined in key tissue samples collected at the time of euthanasia. $TCID_{50}$, 50% tissue culture infectious dose; LN, lymph node. B) Pathology scores for liver and spleen. Scores were generated by using the scoring system described in the Materials and Methods.

### Systemic Host Responses

Differences in systemic host immune responses were monitored in serum of infected animals. Levels of some proinflammatory cytokines increased in later stages of the disease in all animals (Figure 3). Although levels of tumor necrosis factor-α were similar (slightly higher for EBOV-Mayinga–infected animals) for both groups over time (Figure 3, panel A), serum levels of IL-6 increased earlier in the EBOV-Mayinga–infected animals and reached similar levels at time of euthanasia (Figure 3, panel B). The most striking difference was observed for IFN-γ; a >300% increase was observed in EBOV-Makona–infected animals at the time of euthanasia (Figure 3, panel C). Thrombocytopenia is a hallmark of EHF in human and nonhuman primates. Therefore, we monitored levels of sCD40L, a marker for platelet activation. Levels of sCD40L decreased in both groups during the infection (Figure 3, panel D), a fact that correlated well with decreasing platelet levels (Figure 2, panel C). Finally, antiinflammatory marker IL-10 and chemokine monocyte chemotactic protein 1 were found to be upregulated during the course of infection in all the animals, and there were no differences between the 2 groups (Figure 3, panels E, F). Overall, targeted systemic host responses were upregulated for all the animals, and except for IFN-γ, there were minor differences between the 2 groups and among individual animals.

### Pathologic Changes

Animals in both groups showed typical gross pathologic changes for EBOV infections in macaques, and no differences in virus titers were found in tissues collected at the time of euthanasia (Figure 4, panel A). Pathology scores for liver and spleen, key target organs for EBOV infection, were similar (Figure 4, panel B). There were no apparent histopathologic differences between lesions induced by EBOV-Makona or EBOV-Mayinga infections, and both strains caused multifocal to coalescing hepatic necrosis with acute inflammation (Figure 5, upper panels). In addition, there were abundant fibrin microthrombi within the hepatic sinusoids. Likewise, splenic lesions produced by either virus strain were indistinguishable and consisted of necrosis (lymphocytolysis) of the white pulp, abundant fibrin within the red pulp, and multifocal acute inflammation (Figure 5, upper panel). Immunohistochemical analysis showed copious viral antigen in hepatocytes, endothelial cells, and mononuclear cells, which was collocated with hepatic and splenic lesions (Figure 5, lower panels). Overall, lethal EHF developed in all animals, and they showed similar anatomic pathologic features.

## Discussion

EHF with similar hallmarks of the disease developed in all infected cynomolgus macaques, and all animals had to be euthanized according to protocol. However, despite similar onset of symptoms (fever) for all animals, disease progression in the EBOV-Makona–infected animals was delayed, which suggested attenuation in virulence for this strain in macaques. Nevertheless, the macaque model is well suited to determine the efficacy of existing vaccines (historically assessed in cynomolgus macaques) and treatment strategies (historically tested in rhesus macaques) against the currently circulating EBOV-Makona strain in West Africa.

In this study, we sought to establish a macaque disease model for the EBOV-Makona strain currently circulating in West Africa to advance our response capabilities by testing urgently needed countermeasures against this newly emerged virus and by that fulfilling a critical capacity gap. We compared disease progression and pathogenesis of 2 EBOV strains, the prototype EBOV-Mayinga

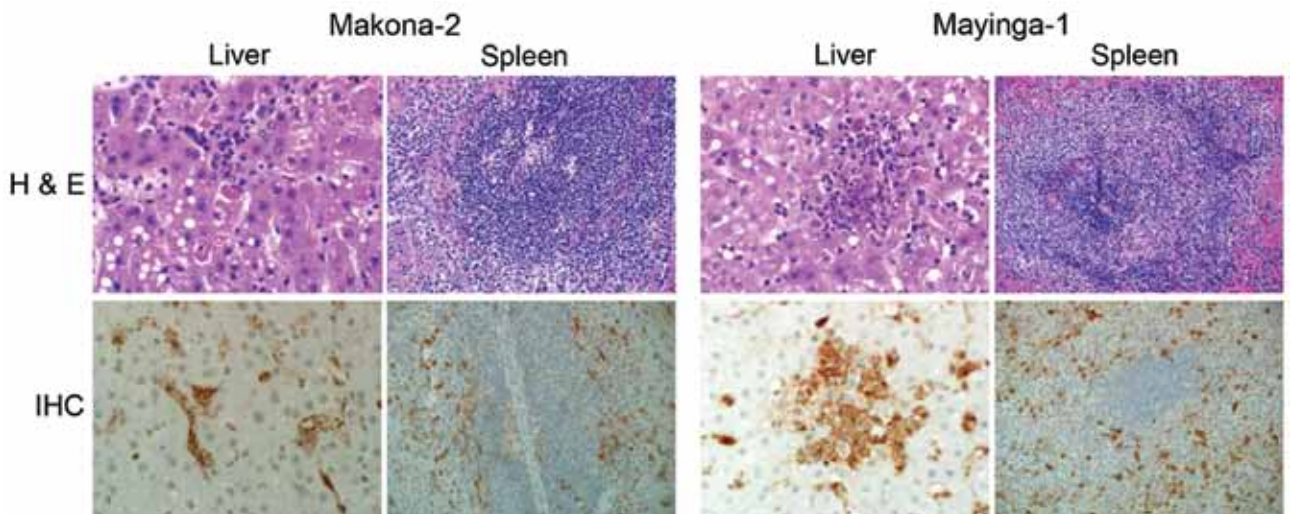


**Figure 5.** Pathologic results for 6 cynomolgus macaques infected with Ebola virus strains Makona or Mayinga. Liver and spleen sections were stained with hematoxylin and eosin (H & E; top panels) and analyzed for necrosis, microthrombi, lymphocytosis, and inflammation. Sections were also stained with a polyclonal rabbit serum against Ebola virus viral protein 40 for detection of viral antigen (immunohistochemical [IHC] analysis; bottom panels). Sections from a representative animal in each group are shown. Original magnification levels: liver, ×40; spleen, ×20.

and the current EBOV-Makona strain (*2*), in cynomolgus macaques. In general, infections in both groups were similar with regard to disease onset, hematology, blood chemistry, systemic host responses, and end-stage viral organ load and pathologic changes. However, EBOV-Makona–infected animals lagged behind in many parameters; the most notable difference was in progression to end-stage disease. Although clinical scores remained similar in the early stage of disease (until day 4 postinfection), EBOV-Makona–infected macaques reached euthanasia criteria on average 2 days later than EBOV-Mayinga–infected animals (7.67 days vs. 5.67 days postinfection, respectively). This finding was associated with less pronounced and more restricted macular cutaneous rash, slightly lower viremia at end-stage disease, less pronounced increases in liver enzyme levels, and slightly delayed systemic proinflammatory and chemokine responses for EBOV-Makona–infected macaques than for EBOV-Mayinga–infected macaques. Overall, virulence of EBOV-Makona seemed to be decreased in this animal model, although severe/lethal EHF eventually developed in all EBOV-infected macaques. The fact that all infections were lethal explains why end-stage pathologic changes were similar in animals infected with both virus strains and why there were virtually no differences in gross pathologic and histopathologic changes in major target organs.

A major distinction is related to IFN-γ levels during end-stage disease with dramatically higher systemic levels for EBOV-Makona–infected macaques. This finding has been reported for an EBOV-Mayinga–infected rhesus macaque (*7*) and was described for lethal EHF cases in humans (*10*). EBOV-Mayinga–infected macaques, which showed faster disease progression, did not show these strikingly increased IFN-γ levels. One can only speculate that ≥7 days (expected time for initiation of adaptive immune responses) after EBOV infection seem to be necessary for adequate stimulation of T cells, the main source of IFN-γ (*11*).

The case-fatality rate in humans in the current outbreak in West Africa (≈50%) does not seem nearly as high as that reported for the initial 1976 outbreak in the former Zaire (now Democratic Republic of the Congo) (≈90%), which suggests lower virulence of this new strain from West Africa (*1*,*3*,*12*,*13*). It is difficult to say if the seemingly lower virulence in humans is reflected in our macaque model because this model remains lethal for all studied EBOV strains, including the current strain in West Africa (EBOV-Makona). However, it seems fair to conclude that virulence of the strain from West Africa in macaques is not increased compared with other EBOV strains. Nevertheless, the macaque model that we developed fills a major capacity gap and can be used for development of urgently needed countermeasures against the new EBOV-Makona strain from West Africa.

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# Effect of Live Poultry Market Closure on Avian Influenza A(H7N9) Virus Activity in Guangzhou, China, 2014

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We assessed the effect of closing live poultry markets in China on influenza A(H7N9) virus detection and viability. Intensive sampling was carried out before, during, and after a 2-week citywide market closure; the markets were cleaned and disinfected at the beginning of the closure period. Swab samples were collected at different sites within the markets and tested for H7N9 by real-time reverse transcription PCR and culture. During the closure, H7N9 viral RNA detection and isolation rates in retail markets decreased by 79% (95% CI 64%–88%) and 92% (95% CI 58%–98%), respectively. However, viable H7N9 virus could be cultured from wastewater samples collected up to 2 days after the market closure began. Our findings indicates that poultry workers and the general population are constantly exposed to H7N9 virus at these markets and that market closure and disinfection rapidly reduces the amount of viable virus.

Influenza A(H7N9) virus emerged in eastern China in March 2013; within 2 years, infections were confirmed in >550 persons and >200 persons had died (*1*). Birds in live poultry markets (LPMs) are considered a major source of H7N9 infection in humans (*2*–*4*). On April 1, 2013, Guangzhou, the capital of Guangdong Province in southern China, implemented surveillance for avian influenza viruses (AIV) in 144 LPMs, in parallel with strengthened surveillance in humans (*5*). Measures included interventions such as daily cleaning, disinfection, and monthly rest days during which poultry were cleared from the markets. Before H7N9 virus infections were identified in humans or poultry in Guangdong Province, the interventions reduced detections of other AIVs by 34% in retail LPMs. (*6*).

When the second epidemic wave of H7N9 virus infection in humans began in October 2013, the virus had spread to China's southern provinces, and Guangdong Province reported the highest number of infections (*7*). However, in Guangzhou, where the LPM interventions were still in place, no cases of H7N9 virus infections in humans were detected until mid-January 2014 (*8*); by mid-February, the case count reached 10 (*5*). In response, the Guangzhou city administration announced a 2-week citywide market closure starting on February 15, during which trading and storing of live poultry were banned in all locations, including retail and wholesale LPMs (*9*). Only sales of frozen poultry were allowed in supermarkets and malls. The Guangzhou Center for Disease Control and Prevention (GZCDC) established enhanced surveillance in addition to the existing routine LPM surveillance to assess its effect on H7N9 virus isolation and survival.

Previous evidence showed that market closures are highly effective in preventing H7N9 virus infections in humans (*10*,*11*) by substantially reducing human exposure to poultry. However, evidence regarding the effect of such closures on AIV activity within the market environment is limited. Such information may better inform the decision to try alternative interventions, such as market rest days or a ban on keeping unsold poultry in LPMs overnight. We assessed the effect of market closure on virus isolation and survival in a natural LPM setting. The study protocol was reviewed and approved by the Research Ethics Committee of GZCDC.

## Materials and Methods

### Collecting and Testing Environmental Samples

Routine and enhanced surveillance were established in LPMs in Guangzhou for long-term AIV monitoring and

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investigation of elevated AIV activity, respectively. Routine surveillance of LPMs was initiated in all 12 districts in Guangzhou on December 26, 2013; a total of 39 randomly selected LPMs were involved. A total of 2–6 retail LPMs from each district and 3 wholesale LPMs from the city were randomly selected; 4–5 environmental swab samples were collected each week from 2–4 randomly selected retail or wholesale stalls. During January 23–30, 2014, additional environmental samples were collected in 4 LPMs immediately before and after poultry were removed and after the markets were disinfected; the samples were tested to assess the effectiveness of the interventions (Figure 1). Swab samples were collected and tested individually.

The citywide LPM closure was implemented during February 15–28, 2014. The LPMs were disinfected once, on February 14, after poultry were removed and the markets thoroughly cleaned. Poultry cages, surfaces of processing tables, and the floor were cleaned with 0.05%–0.1% diluted chlorine solution (expected chlorine concentration 500–1,000 mg/L water). To limit potential market-specific variations in virus activity, enhanced surveillance sites were set up in Panyu district, which comprises one tenth the population of Guangzhou (Figure 1). Samples were collected from retail and wholesale LPMs and from a dressed poultry market (DPM), in which poultry are stocked, processed, and traded differently than they are in LPMs (Table 1). Three retail LPMs, 1 wholesale LPM, and 1 DPM were randomly selected from 77 wet markets in Panyu district. During enhanced surveillance, 12 rounds of intensive sampling were performed before, during, and after the 2-week citywide LPM closure.

During routine and enhanced surveillance, GZCDC collected environmental samples from poultry cages; the inner surface of defeathering machines; chopping boards;

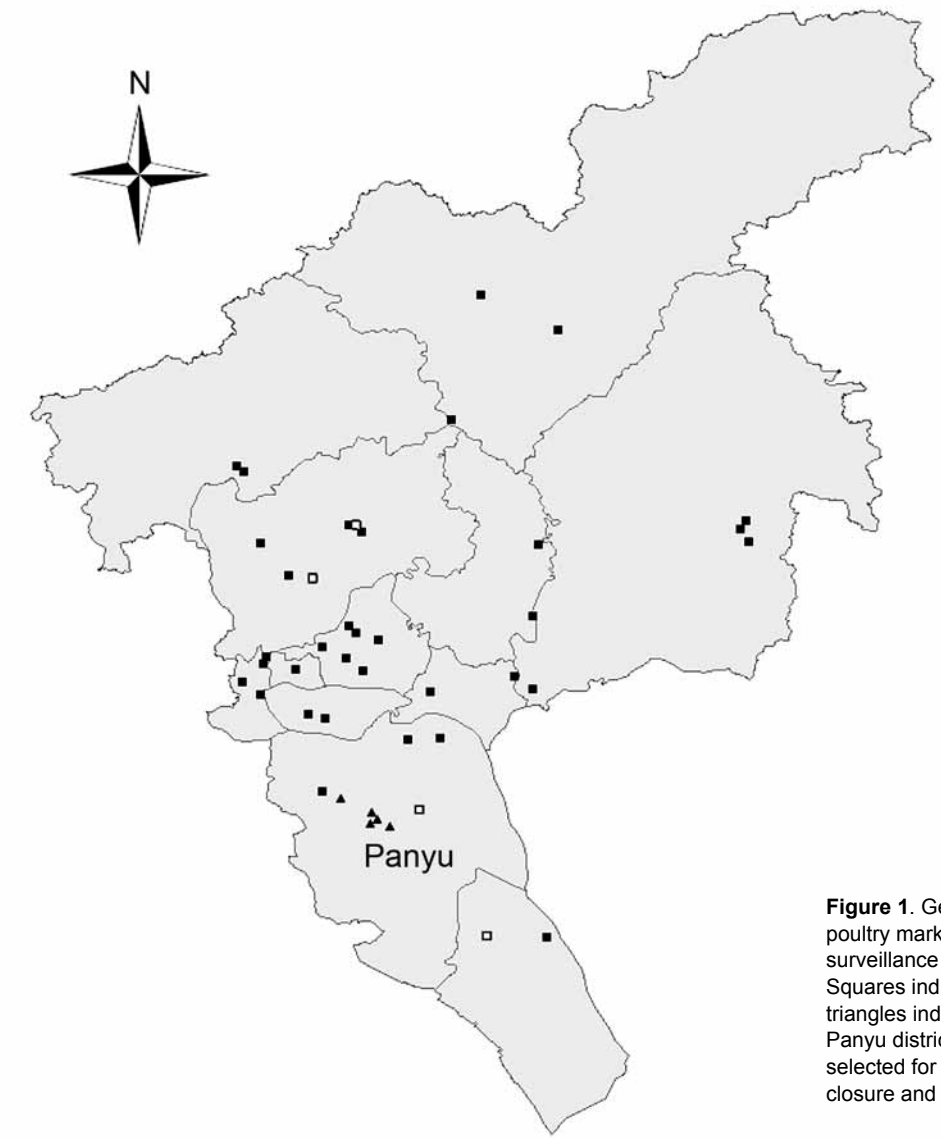


**Figure 1**. Geographic distribution of the live poultry markets under routine and enhanced surveillance in Guangzhou, China, 2014. Squares indicate routine surveillance sites; solid triangles indicate enhanced surveillance sites (in Panyu district); open squares indicate markets selected for comparison before and after market closure and disinfection.

**Table 1.** Characteristics of 5 poultry markets under enhanced surveillance, Guangzhou, Guangdong Province, China, 2014*

| Market characteristic | Live poultry markets | | Dressed poultry market |
|---|---|---|---|
| | Retail | Wholesale | |
| Source of poultry | Wholesale market | Backyard or large farms | Wholesale market |
| Volume of poultry stock | Small | Large | Small |
| Live poultry sold | Yes | Yes | No |
| Size, $m^2$ | 60/50/50† | 3,000 | 25 |
| No. poultry stalls | 6/5/5† | 67 | 5 |
| Approximate no. poultry traded/day | 206/285/112† | 28,640 | 190 |
| On-site slaughtering | Yes | Yes | No |
| Available sampling sites | | | |
| Poultry cage | Yes | Yes | No |
| Defeathering machine | Yes | Yes | No |
| Chopping board | Yes | No | Yes |
| Processing table | Yes | No | Yes |
| Bucket holding poultry meat | No | Yes | No |
| Wastewater | Yes | Yes | Yes |
| Poultry drinking water | No | Yes | No |

*Three retail LPMs, 1 wholesale LPM, and 1 DPM were randomly selected for study from 77 wet markets in Panyu district, where enhanced surveillance was implemented.
†Data are for the 3 retail markets.

surfaces of processing tables; and barrels holding poultry meat, wastewater, and drinking water for chicken. Because each type of LPM has a different setup for poultry processing and sales, environmental samples were collected only from the relevant sampling sites available within the respective LPMs (Table 1).

### Laboratory Procedures

Universal transport medium (Copan Italia, Brescia, Italy) was used to preserve the environmental samples, which were stored in a box with ice packs at 4°C and transported to the laboratory within 4 hours. A QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) was used to extract viral RNA. Influenza A virus M gene and H7N9 virus hemagglutinin (HA) RNA were detected as described previously (*12*) by using a real-time reverse transcription PCR (rRT-PCR) (SuperScript III Platinum One-Step qRT-PCR Kit; Invitrogen, Carlsbad, CA, USA) and H7-specific primers and probe provided by the Chinese National Influenza Center. Samples positive for H7N9 virus by rRT-PCR were inoculated into the allantoic sac of 10-day-old specific pathogen free embryonated chicken eggs and incubated for 48–72 h at 35°C for virus isolation (*13*).

### HA Gene Sequencing and Phylogenetic Analysis

The HA gene of isolated strains was amplified by rRT-PCR, and the products were sequenced by Life Technologies Inc. (Carlsbad, CA, USA) as described previously (*14*). The HA sequences were submitted to GenBank (accession nos. KP326319–KP326321). Reference HA sequences were obtained from GenBank from H7N9 virus strains isolated from eastern and southern China. We performed multiple sequence alignments and constructed the phylogenetic tree with MEGA 6.0.6 (http://www.megasoftware.net) by using a neighbor-joining method with 1,000 bootstrap replicates.

### Statistical Analysis

We calculated rRT-PCR detection rates for H7N9 virus only and for all AIVs from the enhanced surveillance before and after disinfection in 4 markets under routine surveillance by dividing the number of rRT-PCR–positive results by the number of samples tested. Samples were screened by rRT-PCR, and those that were H7N9-positive were further tested by culture if sufficient material was available. Therefore, the H7N9-positive isolation rate at each time point was calculated by multiplying the proportion of rRT-PCR–positive samples by the proportion of culture-positive samples. We assumed a binomial distribution and provided exact 2-sided 95% CIs for the detection rates. We obtained the 95% credible intervals for H7N9 virus-positive isolation rates by using a Bayesian method with the Jeffreys noninformative beta distribution priors for the positive proportions by rRT-PCR and by culture.

We tested the effects of market disinfection and closure on detection rates for H7N9 virus and for all AIVs at different times after these interventions were implemented. Logistic regression models were used, accounting for potential confounders such as specific markets and sampling sites. The effect of market disinfection and closure on H7N9 virus isolation rates was similarly tested, after accounting for missing data from H7N9 virus rRT-PCR–positive samples not available for culture, by using multiple imputation methods with 50 imputed datasets. Given the short study period, limited effects of meteorological variables on virus activity were assumed and not adjusted for in the model. We also compared virus detection rates between the different sampling sites. All statistical analyses were conducted by using R version 3.1.1 (https://www.r-project.org/).

## Results

In the routine surveillance, 214 samples were collected from 4 retail LPMs on the same day immediately before

and after the LPMs were disinfected. Testing showed a moderate decrease in the rates of detection of H7N9 virus and other AIVs by rRT-PCR in each LPM after disinfection (Figure 2). The pooled estimated reduction ratios were 58.0% (95% CI 8.9%–80.6%) for H7N9 virus and 64.2% (95% CI 30.6%–81.5%) for all AIVs.

A total of 1,705 environmental samples were collected from the targeted enhanced LPM sites. Figure 3, panel A shows the detection rates of AIVs, including H7N9 virus, by rRT-PCR before, during, and after the period of the city-wide market closure. Before market closure, site-matched testing (i.e., testing of the same environmental locations between different types of markets) showed the detection rates for H7N9 virus and for all AIVs were significantly higher for retail LPMs than for wholesale LPMs ($p = 0.003$ and $p = 0.032$, respectively). Detection rates for AIVs (including H7N9 virus) were higher in retail LPMs than in the DPM ($p = 0.043$). The samples positive for H7N9 virus by rRT-PCR were cultured for virus isolation (Figure 3, panels B and C).

In the retail LPMs under enhanced surveillance, in the nonintervention period, H7N9 virus isolation rates were <20%, and H7N9 virus and AIV RNA detection rates by rRT-PCR fluctuated at 10%–30% and 20%–60%, respectively. On the first day of market closure, after the markets had been disinfected the preceding night, detection rates for H7N9 virus and all AIVs decreased by ≈50% (Figure 3). During the entire market closure period, RNA detection rates for H7N9 virus and all AIVs decreased by >70% in retail markets, and H7N9 virus isolation rates decreased by >90% (Table 2). After retail LPMs were reopened, H7N9 virus isolation rates increased to a level slightly lower than those before closure.

The wholesale market had low H7N9 virus detection and isolation rates before and during market closure, but rates increased markedly once the markets reopened. In the DPM, detection rates for AIVs decreased substantially during the market closure and increased greatly when the market reopened. In each type of market, detection rates for H7N9 virus and for all AIVs and isolation rates for H7N9 virus quickly rebounded to preclosure or higher levels after the markets reopened, except for H7N9 virus detection and isolation rates in retail markets (Table 2). Detection rates for H7N9 virus and for all AIVs on chopping boards in retail LPMs were considerably higher than rates for other sampling sites (Table 2). During the study period, H7N9 virus was isolated from 23 environmental samples by virus culture. Of the 23 samples, 19 were collected in the nonintervention period, including those collected from all sampling sites in retail LPMs (Table 3). Only 4 virus-positive samples were identified during the market closure period (Figure 3); 3 were collected on the first day. The 4 positive samples were collected from all 3 types of poultry markets;

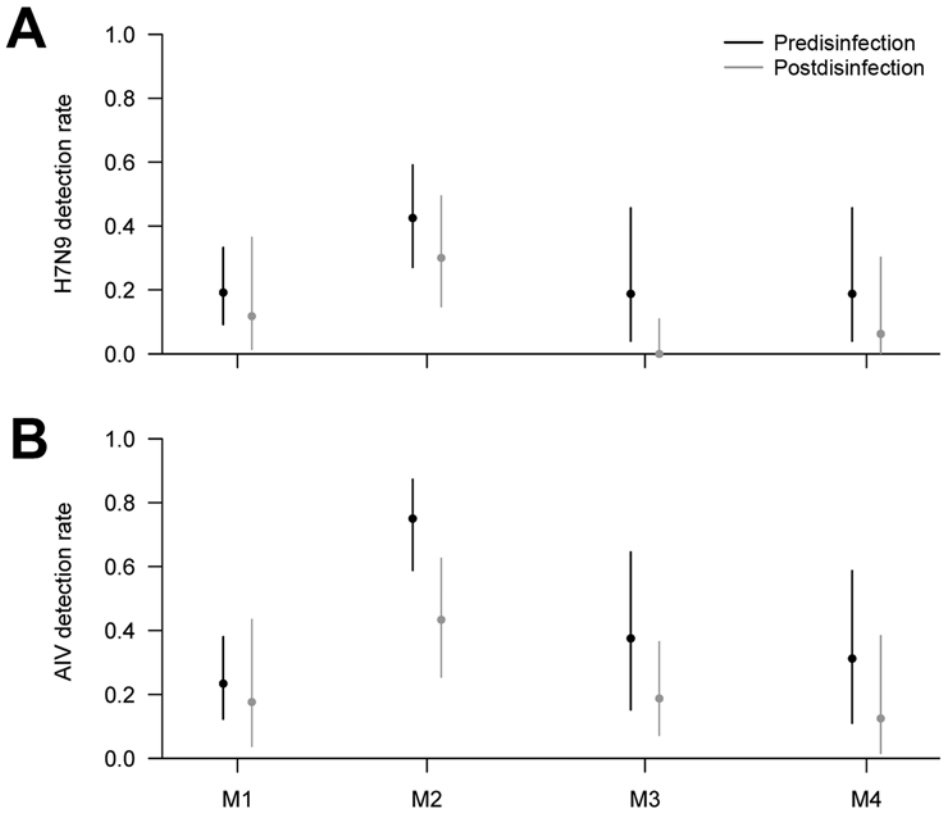


**Figure 2**. Detection rates for A) avian influenza A(H7N9) virus and B) all avian influenza viruses (AIVs) by using real-time reverse transcription PCR before and after disinfection in 4 retail live poultry markets (M1–M4), Guangzhou, China, 2014.

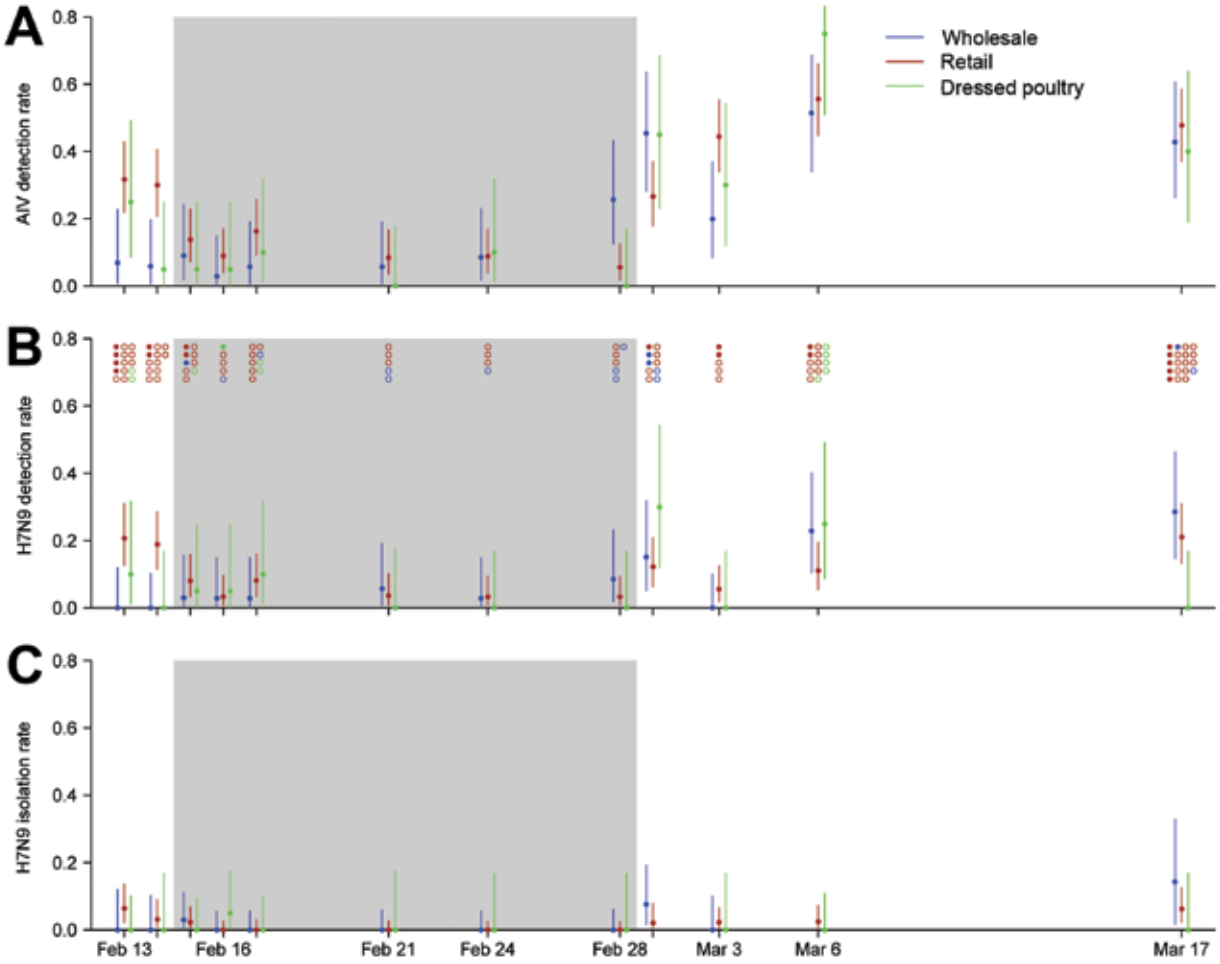


**Figure 3**. Avian influenza virus (AIV) activity in wholesale, retail, and dressed poultry markets under enhanced surveillance in Guangzhou, China, 2014. A) AIV and B) influenza A(H7N9) virus detection rates as determined by real-time reverse transcription PCR (rRT-PCR). Circles at the top of panel B indicate H7N9 virus–positive (solid) and –negative (open) samples isolated by culture from the different types of poultry markets. Some H7N9 virus samples positive by rRT-PCR did not have sufficient sample remaining for virus culture; the numbers of samples cultured and positive at each time point are shown. C) H7N9 virus isolation rates as determined by culture. Rates for positive cultures were estimated on the basis of the samples available for culture, as described in Materials and Methods. Vertical bars indicate 95% CIs for detection rates and 95% credible intervals for isolation rates. Gray shading indicates the 2-week citywide market closure, which began on February 15, 2014.

2 were from wastewater, 1 from a chopping board, and 1 from a defeathering machine.

We sequenced 3 H7N9 virus strains collected from a the same enhanced surveillance retail LPM before, during, and after the market closure. All 3 strains had identical HA genes and differed from a strain collected on January 12 during routine surveillance, A/environment/Guangzhou/1/2014(H7N9), by mutations at 3 sites (D264E, R364K, and K414T). Phylogenetic analysis showed that the strains were genetically closer to the lineage in southern than in eastern China (Figure 4).

## Discussion

We report RNA detection rates for H7N9 virus and for all AIVs as well as viable virus survival in retail and wholesale LPMs and a DPM in Guangzhou before, during, and after market closure and disinfection. Before interventions were implemented on February 15, 2014, detection rates were much higher in retail markets than wholesale markets; this finding is in keeping with the theory that AIVs amplify in retail LPMs (*15*). During normal trading days, H7N9 virus was isolated in retail LPMs from numerous sampling sites, ranging from poultry cages at the back of the retail stalls to the processing tables and chopping boards near the customers. This finding indicates that poultry workers and customers had constant exposure to H7N9 virus in winter, when virus activity is high.

H7N9 virus was infrequently identified on poultry farms after its emergence in 2013. For example, no H7N9 virus was detected in Guangdong farms until March 2014

**Table 2.** Estimated effect of market closure and contaminated environmental sites on AIV and influenza A(H7N9) virus detection in 5 poultry markets under enhanced surveillance, Guangzhou, Guangdong Province, China, 2014*

| | Retail LPMs, aOR (95% CI)† | | | Wholesale LPM, aOR (95% CI) | | | DPM, aOR (95% CI) | |
|---|---|---|---|---|---|---|---|---|
| | rRT-PCR | | H7N9 | rRT-PCR | | H7N9 | rRT-PCR | |
| Variable | AIV | H7N9 | culture | AIV | H7N9 | culture | AIV | H7N9 |
| Period | | | | | | | | |
| Before market closure | Ref | Ref | Ref | Ref | –‡ | –‡ | Ref | Ref |
| During market closure | 0.25 (0.16–0.39) | 0.21 (0.12–0.36) | 0.08 (0.02–0.42) | 1.60 (0.52–4.90) | 0.22 (0.10–0.50) | 0.11 (0.01–0.89) | 0.30 (0.09–0.98) | 0.68 (0.12–3.89) |
| After market closure | 1.78 (1.20–2.63) | 0.58 (0.35–0.95) | 0.73 (0.27–1.98) | 10.3 (3.52–30.3) | Ref | Ref | 5.27 (1.97–14.1) | 3.32 (0.68–16.1) |
| Environmental samples tested | | | | | | | | |
| Poultry cage | Ref | Ref | Ref | Ref | Ref | § | – | – |
| Defeathering machine | 1.15 (0.61–2.14) | 1.66 (0.74–3.70) | 1.25 (0.20–7.87) | 2.49 (1.09–5.68) | 1.21 (0.40–3.65) | § | – | – |
| Chopping board | 2.64 (1.60–4.37) | 2.12 (1.06–4.26) | 3.52 (0.88–14.0) | – | – | – | 0.56 (0.22–1.41) | 3.18 (0.98–10.3) |
| Processing table | 1.16 (0.73–1.85) | 1.15 (0.59–2.25) | 1.09 (0.26–4.67) | – | – | – | Ref | Ref |
| Bucket holding poultry meat | – | – | – | 0.97 (0.38–2.44) | 0.17 (0.02–1.40) | § | – | – |
| Wastewater | 1.60 (0.95–2.67) | 1.23 (0.58–2.62) | 1.41 (0.28–7.14) | 1.38 (0.70–2.73) | 0.91 (0.37–2.22) | § | 1.15 (0.44–3.06) | 1.16 (0.31–4.36) |
| Drinking water | – | – | – | 2.02 (0.44–9.38) | 2.32 (0.40–13.4) | § | – | – |

*AIV, avian influenza virus; aOR, adjusted odds ratio; DPM, dressed poultry market; LPM, live poultry market; ref, reference; rRT-PCR, real-time reverse transcription PCR; –, no samples tested.
†Also adjusted for potential market differences for the 3 retail markets.
‡No influenza A(H7N9) virus was detected before market closure in wholesale markets, and data from this period were excluded from the regression model.
§There were too few H7N9 virus–positive samples by culture in contaminated environmental sites in wholesale markets and DPM overall for us to estimate the effects. A simplified model was used for wholesale markets.

(*16*), well after the first reported local case in a person in August 2013 (*17*). Infection with highly pathogenic H5N1 virus caused severe symptoms in poultry, but infection with low pathogenic H7N9 virus resulted in mild symptoms; thus, the poultry industry had little incentive to identify H7N9 virus–infected poultry. This fact may preclude efficient surveillance on poultry farms. Because the rate of H7N9 virus detection in retail markets is higher than that on poultry farms, LPMs may serve as a better surveillance point for AIV H7N9 virus.

Our findings suggest that chopping boards and wastewater are more sensitive than other LPM environmental sources for the surveillance of AIV activity; this finding is consistent with those of others (*18*,*19*). We also isolated H7N9 virus in defeathering machines in wholesale LPMs. Surveillance programs and disinfection efforts should prioritize these environmental sources of virus contamination. These findings highlight the need to review and strengthen cleaning and disinfection procedures.

After the markets' initial cleaning and disinfection at the commencement of the citywide market closure, RNA for H7N9 virus and for all AIVs were detectable throughout the 14-day market closure period, albeit at lower detection rates than before cleaning and disinfection. However, viable virus could be cultured only from samples collected within 2 days of market closure. This finding demonstrates that detection of viral RNA by rRT-PCR does not necessarily mean presence of infectious virus. Two of the 4 virus isolates obtained after the market closure were collected from wastewater rather than from solid dry surfaces. AIV can survive much longer (≥2 days) in water (*20*) than on environmental surfaces, and LPM workers who clean water containers may have a higher risk for AIV infection (*21*). Thus, wastewater must be removed or efficiently disinfected, and drinking water used by poultry must be removed if interventions such as those used in Guangzhou are to be effective.

We did not quantify the infectious virus load by titration, even when virus could be cultured at the first sampling time point after market closure; thus, it is possible that the virus titer had decreased compared with that during the preintervention period. It is not clear whether virus titers would have been sufficient to initiate reinfection of reintroduced naive poultry. Previous studies on H9N2 virus demonstrated substantial reduction in isolation rates after a ban on keeping poultry overnight at LPMs, suggesting that transmission can be interrupted by emptying the market overnight (*22*).

We did not collect samples during days 3–5 days after implementation of the interventions. However, the estimated reduction in H7N9 virus and other AIV RNA detection rates by rRT-PCR within a day of the citywide market closure was ≈50% from enhanced and routine surveillance (Figure 2).

**Table 3.** Influenza A(H7N9) virus identified in or on different environmental sites in 5 poultry markets under enhanced surveillance, Guangzhou, Guangdong Province, China, 2014*

| | No. samples/no. tested (%) | | | | | |
|---|---|---|---|---|---|---|
| | Retail LPMs | | Wholesale LPM | | DPM | |
| Environmental sites | rRT-PCR–positive | Culture-positive† | rRT-PCR–positive | Culture-positive† | rRT-PCR–positive | Culture-positive† |
| Before market closure and after market reopened | | | | | | |
| Poultry cage | 12/94 (12.8) | 2/9 (2.8) | 7/56 (12.5) | ‡ | – | – |
| Defeathering machine | 11/59 (18.6) | 2/10 (3.7) | 4/30 (13.3) | 1/3 (4.4) | – | – |
| Chopping board | 18/95 (18.9) | 6/14 (8.1) | – | – | 4/26 (15.4) | 0/1 |
| Processing table | 25/189 (13.2) | 4/18 (2.9) | – | – | 6/60 (10.0) | 0/4 |
| Bucket holding poultry meat | – | – | 1/29 (3.4) | ‡ | – | – |
| Wastewater | 13/95 (13.7) | 2/11 (2.5) | 10/80 (12.5) | 2/3 (8.3) | 3/34 (8.8) | 0/2 |
| Drinking water | – | – | 1/6 (16.7) | ‡ | – | – |
| During market closure | | | | | | |
| Poultry cage | 2/91 (2.2) | 0/2 | 3/59 (5.1) | 0/3 | – | – |
| Defeathering machine | 4/58 (6.9) | 0/4 | 2/30 (6.7) | 1/2 (3.3) | – | – |
| Chopping board | 9/96 (9.4) | 1/8 (1.2) | – | – | 3/27 (11.1) | 0/3 |
| Processing table | 7/188 (3.7) | 0/7 | – | – | 0/59 | – |
| Bucket holding poultry meat | – | – | 0/30 | – | – | – |
| Wastewater | 4/92 (4.3) | 1/4 (1.1) | 3/83 (3.6) | 0/3 | 1/33 (3.0) | 1/1 (3.0) |
| Drinking water | – | – | 1/6 (16.7) | 0/1 | – | – |

*DPM, dressed poultry market; LPM, live poultry market; rRT-PCR, real-time reverse transcription PCR; –, no samples collected.
†Because not all positive samples from rRT-PCR were available for virus culture, isolation rates were derived by using the product of the percentage of rRT-PCR–positive samples and the percentage of those samples that were also culture-positive.
‡These positive samples from rRT-PCR did not have sufficient material available for virus culture.

We observed that all 3 types of poultry markets were recontaminated by H7N9 virus and other AIVs; immediately after markets were reopened, detection rates were as high as those before market closure. In contrast to the preintervention period, during the postintervention period, we found no difference in virus detection rates in the retail and wholesale markets. The reason for this is unclear. It is possible that the sudden closure of the LPMs led to a backlog of poultry in other temporary holding facilities, where mixing of poultry originating from different areas could have led to virus amplification before the poultry reentered the wholesale market. Aside from within-market interventions, tightening controls on poultry or shortening transportation time along the supply chain may be needed to further reduce virus load in LPMs. For the DPM, poultry may have been stored temporarily and prepared elsewhere to preserve freshness, which may have contributed to the unexpectedly high detection rates in this market.

Market rest days, along with a series of other control strategies, have been shown to reduce circulation of low pathogenic AIV in the retail LPM setting (*22*). Compared with other species commonly traded in LPMs (i.e., ducks, geese, and pigeons), chickens and quail were found to be more susceptible to H7N9 virus and shed higher levels of virus for a longer period (*23*). Because they are more susceptible to H7N9 virus, segregating chickens and quail from other species may limit virus transmission in retail and wholesale markets. More studies are needed to understand why LPMs were contaminated by H7N9 virus soon after they were reopened. If recontamination was due to off-site holding of multiple consignments of poultry in ad hoc storage areas, measures must be taken to minimize the need for such storage; well planned, preemptive interventions should replace reactive ones to which the poultry industry cannot rapidly adjust.

Although market closure has been demonstrated to be effective in reducing influenza infections in humans in China (*10*,*11*), its frequent or prolonged implementation may not be sustainable for the poultry industry, even if limited to winter when AIV activity is high. Furthermore, H7N9 virus RNA can also be detected in LPMs during summer (*24*), and such detections may trigger market closures. The general public in China tends not to favor the centralized slaughter of poultry, especially because poultry workers, whose income is disrupted and who experience other economic losses when markets are closed, object to the idea (*25*). Whether an approach that includes interventions such as species segregation, stringent testing at the wholesale market level, frequent cleaning and disinfection of markets, and regular market rest days may reduce the infection risk to a minimal but sustainable level remains to be investigated (*22*,*26*).

Poultry workers in China still demonstrate relatively low awareness of the risk for H7N9 virus infection and compliance with measures to prevent virus transmission (*27*). A serologic study in southern China showed that 54% (52/96) of poultry workers had seroconverted for H7N9 virus during May–December 2013 (*28*), although few cases were virologically confirmed. Among persons in whom cases of H7N9 infection were laboratory confirmed, >50% (43/84) had visited LPMs but only 5% (6/123) had occupational exposure to poultry (*29*). Risk for infection with H7N9 virus for the general public seems to be different from that for poultry workers, who have prolonged and direct exposure to poultry.

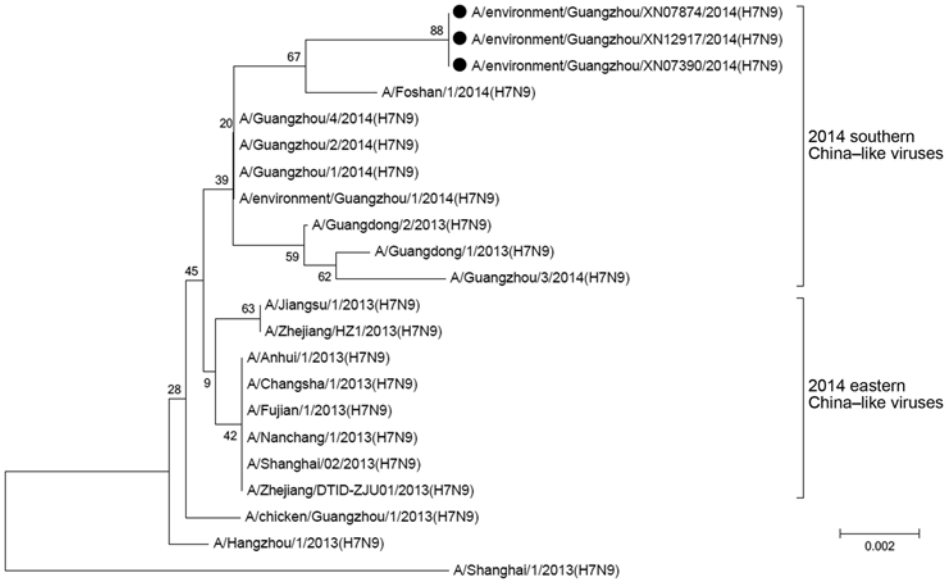


**Figure 4**. Phylogenetic analysis of hemagglutinin genetic sequences of an influenza A(H7N9) virus isolated from an environmental sample collected at a retail live poultry market under enhanced surveillance, Guangzhou, China, 2014. Black circles indicate 3 strains collected before (February 13), during (February 15), and after (March 17) a 2-week market closure. Scale bar indicates base substitution per site.

Such discordance between potential exposure and disease is also noted with H5N1 virus infection and may reflect heterogeneity of host susceptibility to infection with these AIVs (*30*). Although limited human-to-human transmission of H7N9 virus among close contacts has been reported (*14*,*31*), the main transmission route seems to be associated with exposure to LPMs. Hence, special attention should be paid to the LPM environment, which provides the interface between poultry and the general public. For example, chopping boards, which are usually located at the front of the retail stalls, had higher isolation rates of H7N9 virus. Interventions such as adding a screen between customers and the chopping board may reduce the public's exposure to H7N9 virus.

Our study has some limitations. First, the unexpected timing of the poultry market closure shortened the preintervention baseline period and the enhanced surveillance period during which we could obtain samples from LPMs. Thus, the preintervention data may not fully reflect the normal situation, especially in wholesale markets (Figure 3). However, enhanced surveillance showed consistent and substantial reductions in H7N9 virus detection in retail LPMs and the DPM as estimated from those before and after disinfection by routine surveillance. The estimated effect of market closure on H7N9 virus activity should be unbiased and most relevant to the general public, who are primarily exposed to poultry at retail LPMs and DPMs. Second, environmental and poultry samples were not collected in parallel and along the supply chain; thus, we could not identify potential interactions between retail and wholesale markets during the closure period or between poultry and the market environment during trading days. Whether the unexpected market closure led to unusual holding of poultry off site and whether this may have contributed to the postintervention rebound of virus in the markets is unclear. If such interventions are well planned and anticipated, poultry farmers can adjust their shipments to the wholesale market, and this rebound may be avoidable. Third, detection of H7N9 virus RNA does not directly translate into risk for transmission of the virus; transmission depends on multiple factors, such as virus viability (as assessed by virus isolation), infectious virus load, and mode and frequency of contact with different market environments. We did examine H7N9 virus isolation and RNA detection from different sampling sites and provide an overview of H7N9 virus contamination at different time points after market closure and from different environments, which supply an evidence base for fine-tuning current market interventions.

We document the effect of market closures on survival of H7N9 virus in a natural LPM setting. Market closure and disinfection reduced H7N9 viral RNA contamination in the LPM environment by >70% and infectious virus by >90%. However, live virus could be detected for ≈2 days after the intervention, especially in wastewater sources, and H7N9 virus activity returned quickly to preintervention levels once markets reopened. The reason for this rebound requires further investigation to inform the design of more effective interventions. Given limited support from the general public for permanent closure of LPMs (*32*), more sustainable alternative approaches should be considered to minimize the risk of transmission of H7N9 virus from retail LPMs. These approaches might include improving the design of retail stalls, segregating or banning poultry species with high susceptibility to AIVs, scheduling market rest days so that poultry farmers can

adjust shipments, and improving viral surveillance. At the same time, unintended consequences of interventions, such as unauthorized movement of poultry from a closed market to a different trading area, should be avoided (*33*). To strike a balance between minimizing the risk of virus transmission to humans and the demands for live poultry from the public and the interests of the poultry industry, public health and veterinary sectors should strengthen their coordination under a One Health approach (*34*). Clarification of H7N9 virus prevalence along the poultry supply chain (from farm to retail markets), identification of key settings for virus amplification, and characterization of poultry trading patterns during normal and epidemic periods with various interventions will help in preparing an optimal control strategy.

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# Human Infection with *Ehrlichia muris*–like Pathogen, United States, 2007–2013[1]

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An *Ehrlichia muris*–like (EML) pathogen was detected among 4 patients in Minnesota and Wisconsin during 2009. We characterized additional cases clinically and epidemiologically. During 2004–2013, blood samples from 75,077 patients from all 50 United States were tested by PCR from the *groEL* gene for *Ehrlichia* spp. and *Anaplasma phagocytophilum*. During 2007–2013, samples from 69 (0.1%) patients were positive for the EML pathogen; patients were from 5 states: Indiana (1), Michigan (1), Minnesota (33), North Dakota (3), and Wisconsin (31). Most (64%) patients were male; median age was 63 (range 15–94) years; and all 69 patients reported likely tick exposure in Minnesota or Wisconsin. Fever, malaise, thrombocytopenia, and lymphopenia were the most common symptoms. Sixteen (23%) patients were hospitalized (median 4 days); all recovered, and 96% received doxycycline. Infection with the EML pathogen should be considered for persons reporting tick exposure in Minnesota or Wisconsin.

Ehrlichiosis and anaplasmosis are emerging tickborne zoonoses caused by *Ehrlichia* spp. and *Anaplasma phagocytophilum*, respectively (*1–4*). These gram-negative obligate intracellular bacteria infect leukocytes and cause febrile illness in humans (*1*,*3*). In the United States, most infections occur during May–August, months with peak human exposure to hard tick vectors (*3*). The signs, symptoms, and clinical course of ehrlichiosis and anaplasmosis are similar; >50% of patients have fever, headache, chills, malaise, myalgia, and nausea, the most common signs and symptoms (*5*). Vomiting, diarrhea, cough, arthralgia, and confusion are less frequently reported (*3*,*6*). Rashes are infrequently reported among patients with anaplasmosis, but ≈60% of children and 30% of adults with ehrlichiosis report rash (*7*). Leukopenia, thrombocytopenia, and elevated alanine aminotransferase, aspartate aminotransferase, or alkaline phosphatase are common laboratory findings (*3*). Severe complications of anaplasmosis and ehrlichiosis are rare and may include renal failure, pneumonia, acute respiratory distress syndrome, neurologic disorder, and intravascular coagulation (*8*,*9*). Hospitalization rates are high (ehrlichiosis 49% and anaplasmosis 36%) (*1*); fatality rates are <1% among patients with anaplasmosis and 1.8% among patients with ehrlichiosis (*7*,*10*). Elderly and immunocompromised patients, including those with HIV infection and those receiving immunosuppressive therapy for malignancies, are at greatest risk for severe disease (*3*).

Anaplasmosis is reported primarily in temperate regions of North America, Europe, and Asia (*8*,*9*). In the

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United States, *A. phagocytophilum* infects granulocytes and is primarily transmitted by the bite of infected *Ixodes scapularis* and *I. pacificus* ticks. Anaplasmosis occurs in regions where these vectors are prevalent, principally the Northeast and upper Midwest but also Mid-Atlantic and north-central states and, less frequently, the Pacific Northwest. In contrast, human ehrlichiosis is caused primarily by *E. chaffeensis*, which infects monocytes, and less often by *E. ewingii*, which infects granulocytes. Both *Ehrlichia* spp. are transmitted through the bite of an infected *Amblyomma americanum* tick and are found in regions where this vector is prevalent, primarily the Southeast and south-central and Mid-Atlantic states. These 2 *Ehrlichia* spp. were thought to be the only causes of ehrlichiosis in the United States until 2009, when an *Ehrlichia* sp. closely related to *E. muris* was detected in 3 symptomatic patients in Wisconsin and 1 in Minnesota (*11*). This *E. muris*–like (EML) pathogen was also detected among *I. scapularis* ticks in Minnesota and Wisconsin, suggesting that this species may be a potential vector (*11*,*12*; Minnesota Department of Health, University of Wisconsin-Madison Department of Entomology, unpub. data). Testing for ehrlichiosis was not routine in these 2 states because the illness was not thought to be endemic. However, increased recognition and testing for the EML pathogen during 2009–2013 and retrospective review of PCR records during 2004–2008 resulted in identification of 69 cases, including the initially reported 4 cases (*11*). We report the clinical and epidemiologic features of EML human infections detected during 2004–2013 in the United States.

## Methods

### Real-Time PCR Testing

During January 1, 2004–December 31, 2013, real-time PCR testing was performed on DNA extracted from EDTA whole blood samples for *A. phagocytophilum, E. chaffeensis, E. ewingii*, and the EML pathogen by using a modified version of a multiplex PCR assay targeting a conserved region of the *groEL* heat-shock protein operon (*13*) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/10/15-0143-Techapp.pdf). The reaction mix was prepared by using the LC FastStart DNA Master Hybridization Probes Kit (Roche, Indianapolis, IN, USA) with the following final concentrations of reagents: 3 mmol/L MgCl2, 0.5 μM each of the primers, 0.2 mmol/L each of fluorescein-labeled probes and 0.4 mmol/L of LC460-labeled probe. Amplification parameters that used the LightCycler 2.0 thermocycler (Roche) were denatured at 95°C for 10 min followed by 45 cycles of 95°C for 10 s at 20°C/s slope, 55°C for 15 s at 20°C/s slope, and 72°C for 15 s at 20°C/s slope. Melting curve analysis was performed at 95°C for 0 s at 20°C/s slope, 40°C for 60 s at 20°C/s slope, and 85°C for 0 s at 0.2°C/s slope with continuous fluorescence acquisition. Differentiation between organisms was determined by melting temperature analysis, with the EML pathogen producing results in the range of 51.5°C–53.5°C.

For available samples that tested positive by PCR for the EML pathogen during 2009–2013, confirmatory sequence analysis was performed by using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by using Sequencher DNA sequence analysis software version 4.2 (Gene Codes Corporation, Ann Arbor, MI, USA). PCR melting temperature data from LightCycler computer records before 2009 were also retrospectively reviewed to identify cases of EML pathogen infection.

PCR tests were performed at Mayo Medical Laboratories (MML), (Rochester, MN, USA) during 2004–2013, at MML New England (Andover, MA, USA) during 2010–2013, at the Mayo Clinic Health System (Eau Claire, WI, USA) during 2009–2013, and at Marshfield Clinic Laboratories (Marshfield, WI, USA) beginning in 2013. MML is an international reference laboratory with *Ehrlichia* and *Anaplasma* PCR testing sites in Minnesota and Massachusetts, which receive samples from all 50 United States. The Mayo Clinic Eau Claire and Marshfield Clinic sites test samples primarily from Wisconsin residents.

### Acute Phase Serologic Testing and Peripheral Blood Film Examination

Patient serum samples were tested for IgG-class antibodies reacting to *A. phagocytophilum* and *E. chaffeensis* at the Mayo Clinic, as previously described (*11*). Test results with reciprocal titers ≥64 were considered positive. Acute-phase samples were defined as those collected within 10 days of illness onset, and convalescent-phase samples were defined as samples collected >10 days after symptom onset. Conventional thin blood films created from selected patient samples were stained with Wright-Giemsa and examined for evidence of intraleukocytic morulae.

### Patient Information

Demographic, clinical, and epidemiologic information was obtained from health care providers of patients whose samples tested positive by PCR for the EML pathogen; standardized case report forms from the Minnesota and Wisconsin health departments were used to collect data. State health departments obtained and reviewed medical records for hospitalized patients or those treated in an emergency department. Patients were also interviewed by local and state health department staff, who used an investigation questionnaire to collect additional clinical information and travel and tick exposure history.

## Results

### Real-Time PCR Testing and Sequence Analysis

During 2004–2013, of 75,077 patient samples tested by MML (n = 63,185), Mayo Clinic Health System-Eau Claire (n = 5,722), and Marshfield Clinic Laboratories (n = 6,170), blood samples from 69 patients tested positive for the EML pathogen, including 4 previously reported patients (*11*). Sequence analysis of the *groEL* gene was performed for 64 available samples, and results showed that the amplified regions had 100% homology to each other and 98% homology to *E. muris*, thus confirming identification as the EML pathogen.

Of the 69 positive results, 64 (93%) were detected among 39,981 samples submitted from health care providers in Minnesota (33 positive results) and Wisconsin (31 positive results) (Figure 1). Another 5 positive results (3 from North Dakota, 1 from Indiana, and 1 from Michigan) were detected among 35,096 samples submitted from the other 48 states. All 69 patients with positive results were tested during 2007–2013 and reported likely tick exposures in Minnesota or Wisconsin. No cases were detected before 2007.

Among serum samples submitted for *Ehrlichia* and *Anaplasma* PCR, 16,805 were submitted by providers in New England and Mid-Atlantic states that have high reported rates of human anaplasmosis (≥3.0 cases/1 million persons annually for each state of Connecticut, Massachusetts, Maine, Rhode Island, and New York) (*1*). However, the EML pathogen was not detected in samples from these states.

After the EML pathogen was first recognized in the laboratory and alerts were issued to physicians by the Minnesota and Wisconsin state health laboratories, detection of the pathogen among Minnesota and Wisconsin residents was 7–23 (0.1%–0.3%) positive samples per year during 2010–2013 (Table 1). Retrospective review of computer PCR records identified 3 more cases from 2007–2008 and 1 more case from 2009 (not included in the initial publication) (*11*); these cases were identified on the basis of a melting temperature in the EML pathogen range. Archived samples were not available for these 4 patients; therefore, additional laboratory testing was not possible. The EML pathogen was rarely detected compared with detection of *A. phagocytophilum*. During 2013, only 17 (0.12%) of 13,639 samples from Minnesota and Wisconsin were positive for the EML pathogen, compared with 503 (3.7%) samples that were positive for *A. phagocytophilum*.

### Acute Phase Serologic Testing and Peripheral Blood Film Examination

Of the 69 patients with samples testing positive by PCR for the EML pathogen, samples from 6 were tested at a

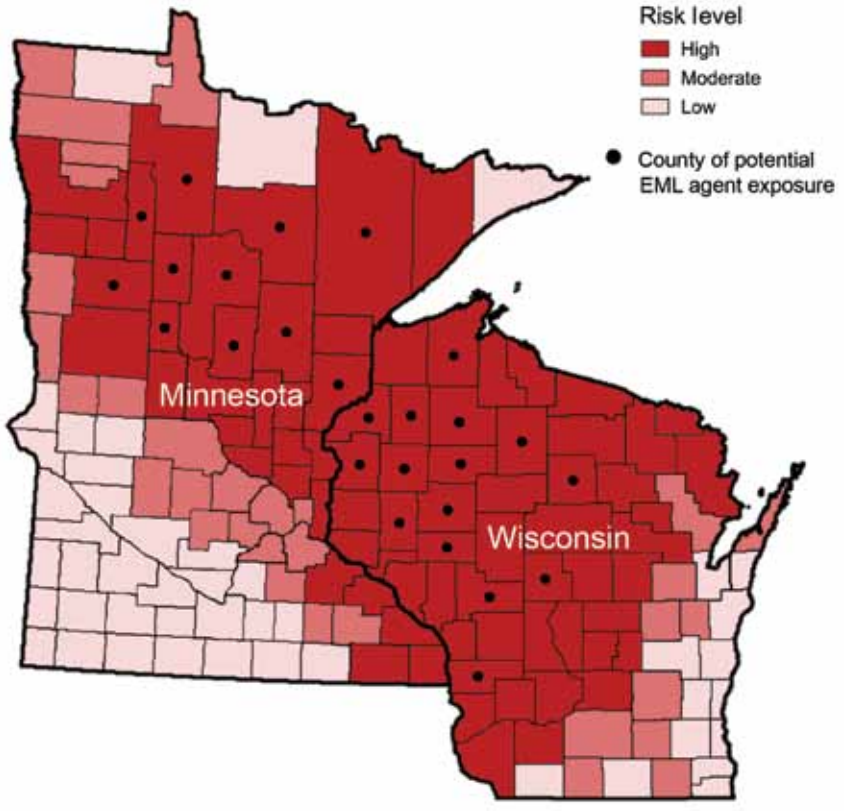


**Figure 1.** Geographic distribution of the likely county in Minnesota or Wisconsin in which exposure to the *Ehrlichia muris*–like (EML) pathogen occurred in relation to the risk for Lyme disease, babesiosis, and anaplasmosis. The risk of tickborne disease is based on county-specific mean annual reported incidence of confirmed Lyme disease and confirmed and probable human anaplasmosis and babesiosis cases in Minnesota and Wisconsin during 2007–2013. Counties with ≤10 cases/100,000 population were classified as low risk; counties with 10–24.9 cases/100,000 population were classified as moderate risk; and counties with ≥25 cases/100,000 population were classified as high risk.

**Table 1.** Number of real-time PCR tests to detect *Ehrlichia* species/*Anaplasma phagocytophilum* performed by year and detection of the EML pathogen among samples from residents of Minnesota and Wisconsin, 2004–2013*

| Testing | 2004† | 2005 | 2006 | 2007 | 2008 | 2009 | 2010 | 2011 | 2012 | 2013 |
|---|---|---|---|---|---|---|---|---|---|---|
| No tests performed | 275 | 520 | 779 | 1,224 | 1,909 | 2,198 | 4,365 | 7,349 | 7,723 | 13,639 |
| No. (%) EML positive | 0 (0) | 0 (0) | 0 (0) | 2 (0.2) | 1 (0.1) | 5 (0.2) | 9 (0.2) | 23 (0.3) | 7 (0.1) | 17 (0.1) |

*Numbers of patients with EML-positive samples do not include 5 EML-positive samples submitted from patients outside MN and WI (2009–2013) who likely acquired infection in MN and WI. EML, *Ehrlichia muris*–like; †First year that PCR assay was used for clinical testing.

commercial laboratory for IgG-class antibodies to *E. chaffeensis* and *A. phagocytophilum* (Table 2; online Technical Appendix Table 2). For 1 patient, an acute sample collected 6 days after illness onset (reciprocal titer 512) and a convalescent sample collected 56 days after illness onset (reciprocal titer 1,024) tested positive for IgG-class antibodies to *E. chaffeensis*. For another patient, an acute sample collected 2 days after illness onset (reciprocal titer 64) tested positive for IgG-class antibodies to *A. phagocytophilum* (online Technical Appendix Table 2). For each of 15 patients whose samples tested positive by PCR for the EML pathogen, 2 thin blood films were examined by using light microscopy with oil immersion. No intraleukocytic morulae were identified in these films.

### Clinical and Epidemiologic Features

Among the 69 EML-positive patients, 44 (64%) were male; age range was 15–94 (median 62) years (Table 2). Patients reported symptom onset during April–December, with peak onset (52%) occurring in June and July (Figure 2). Thirteen (27%) of 49 patients with known immune status had immunocompromised conditions resulting from immunosuppressive therapies: 7 with solid organ allografts received immune modulating pathogens; 2 received chemotherapy to treat malignancy; and 4 received systemic steroids to treat an autoimmune disease (rheumatoid arthritis [n = 3] and mixed connective tissue disease [n = 1]). The most frequently noted signs and symptoms were fever (87%), malaise (76%), headache (67%), and myalgia (60%). Frequently noted laboratory findings among those tested included thrombocytopenia (67%), lymphopenia (53%), leukopenia (39%), anemia (36%), and elevated aspartate aminotransferase or alanine transaminase (78%) (Table 2). Two of 28 patients tested had positive serologic results for *Borrelia burgdorferi*.

The range of days from symptom onset to PCR testing was 0–145 (median 4) days; 59 (86%) of 69 cases were detected within 10 days, and 94% were detected within 21 days. One patient reported new onset of fatigue and sweats ≈4 months before testing, but because of a complex medical history, a more compressed period for EML infection could not be excluded. The hospitalization rate was 23% (16/69 patients), and length of stay was 2–15 (median 4) days (Table 2). All patients recovered. Of 68 patients with available treatment information, 66 (97%) received a course of doxycycline and 2 recovered without therapy.

## Discussion

We report clinical and epidemiologic data for 69 symptomatic patients infected with the newly described EML pathogen, including 4 patients with previously reported cases (*11*). All patients reported likely exposure to ticks in Minnesota or Wisconsin, and the EML pathogen was not detected in >16,000 patient samples from other US states that have high incidence of diseases caused by other tick-borne pathogens transmitted by *Ixodes* spp. ticks. The relatively limited area where EML infections were likely acquired suggests that the EML pathogen may have a limited geographic distribution. This potential geographic focus is also supported by tick data in which the EML pathogen has been detected only among *I. scapularis* ticks from Minnesota and Wisconsin and not from *I. scapularis* and *A. americanum* ticks from other US states (Minnesota Department of Health; University of Wisconsin–Madison Department of Entomology; and US Army Department of Defense Human Tick Test Kit Program, unpub. data) (*12*,*14*). Additional testing of humans and ticks from regions outside the upper midwestern United States would be valuable for further defining the distribution of the EML pathogen.

Data accumulated to date indicate that the epidemiologic features and clinical signs and symptoms of infection with the EML pathogen are similar to those of anaplasmosis and ehrlichiosis caused by other pathogens. Illness onset peaked in June and July, consistent with infection occurring during peak *I. scapularis* nymph activity of May and June in Minnesota and Wisconsin. Infected patients were more frequently male (1.8:1), and average patient age was 61 years. Fever, malaise, headache, and myalgia were the most common symptoms; leukopenia, lymphopenia, thrombocytopenia, and elevated levels of hepatic transaminase were the most common laboratory findings, consistent with anaplasmosis and ehrlichiosis caused by *E. chaffeensis* and *E. ewingii* (*1*,*3*,*15*). No intraleukocytic morulae were identified on peripheral blood films, suggesting that this method may not be sensitive enough to detect this infection. The type of leukocyte infected by the EML pathogen is unknown.

Among 49 patients with reported immunocompromised status, 13 (27%) were receiving systemic immunosuppressive therapy. This percentage of patients with immunocompromising conditions is higher than that previously reported among patients with anaplasmosis or other causes of ehrlichiosis (*1*,*8*). Consequently, immunocompromised patients may be particularly susceptible to infection with the

EML pathogen. Most (7/13) immunosuppressed patients were solid organ allograft recipients; others were receiving immunosuppressive therapies for malignancy or autoimmune conditions. Two of 28 patients for whom information was available had positive serologic tests for *B. burgdorferi*. These results highlight the possibility of co-infection with other tickborne pathogens, an unexpected occurrence if *I. scapularis* ticks are the primary vector of the EML pathogen.

**Table 2.** Clinical features and laboratory findings among patients infected with the EML pathogen, United States, 2007–2013*

| Clinical features | Value |
|---|---|
| Patient age at illness onset, y | |
| Range | 15–94 |
| Mean | 60.8 |
| Median | 62 |
| Sex† | |
| M | 44/69 (64%) |
| F | 25/69 (36%) |
| Period from symptom onset to testing, d | |
| Range | 0–145 |
| Mean | 9.5 |
| Median | 4 |
| Immunocompromised state† | 13/49 (27%) |
| Solid organ allograft recipient | 7 |
| Receipt of chemotherapy for malignancy | 2 |
| Receipt of systemic steroids for autoimmune disease‡ | 4 |
| Patient symptoms† | |
| Fever | 60/69 (87%) |
| Malaise/fatigue | 47/62 (76%) |
| Headache | 46/69 (67%) |
| Myalgia | 41/68 (60%) |
| Nausea/vomiting | 15/69 (22%) |
| Rash | 8/69 (12%) |
| Laboratory findings†§ | |
| Anemia | 18/50 (36%) |
| Leukopenia | 20/51 (39%) |
| Lymphopenia | 17/32 (53%) |
| Thrombocytopenia | 34/51 (67%) |
| Elevated AST or ALT | 18/23 (78%) |
| *Ehrlichia chaffeensis* positive serology, acute | 1/6 (17%) |
| *Anaplasma phagocytophilum* positive serology, acute | 1/6 (17%) |
| *Borrelia burgdorferi* positive serology or PCR | 2/28 (7%) |
| Doxycycline treatment†¶ | 66/68 (96%) |
| Length of treatment, d | |
| Range | 7–30 |
| Mean | 15.0 |
| Median | 14 |
| Hospitalization†# | 16/69 (23%) |
| Length of stay, d | |
| Range | 2–15 |
| Mean | 6.1 |
| Median | 4 |
| Immunocompromised patients† | 10/15 (67%) |
| Death† | 0/69 (0%) |

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; EML, *Ehrlichia muris*–like.
†Expressed as number of patients with a specific risk factor, symptom, laboratory finding, or outcome, divided by the number of patients with available data. Corresponding percentage is also provided.
‡Of the 4 patients receiving systemic steroids for an autoimmune condition, 3 had rheumatoid arthritis, and 1 had mixed connective tissue disease.
§Anemia, hemoglobin <13.5g/dL for male or <12.0g/dL for female patients; leukopenia, <3.5 cells x $10^{-9}$/liter; thrombocytopenia, <150 cells $\times$ $10^{-9}$/liter; elevated AST, >48 U/liter; elevated ALT, >55 U/liter; positive *Ehrlichia chaffeensis* or *Anaplasma phagocytophilum* serology, reciprocal IgG titer $\geq$64.
¶Two patients recovered without treatment. Treatment information was unavailable for 1 patient.
#Length of stay was unknown for 2 of the 16 hospitalized patients.

Commercial serologic testing of samples from 2 patients with EML infection showed positive IgG antibody results to *E. chaffeensis* or *A. phagocytophilum*. This finding supports data from a previous study that suggests that cross-reactivity can occur between *A. phagocytophilum* and *Ehrlichia* spp. (*16*). Additional serologic studies are needed to determine how frequently cross-reactivity occurs between the EML pathogen and other *Ehrlichia* spp. This information would help assess whether current commercially available *E. chaffeensis* serologic tests are reliable for testing for evidence of EML infection.

The population tested during our study does not necessarily represent the population of infected patients but represents patients who sought medical evaluation for their illnesses and had blood submitted for *Ehrlichia* and *Anaplasma groEL* PCR testing at Mayo Clinic or Marshfield Clinic laboratories. The 75,077 samples do not necessarily represent individual patients because multiple samples could have been submitted from some patients. The MMLs began testing for the EML pathogen during 2004. The absence of detection of the EML pathogen in human samples before 2007 is likely related to the small number of tests performed during that period and may indicate a low prevalence of EML infections in humans. However, detection of *E. muris* sequences that closely resemble the EML DNA pattern in archived *I. scapularis* ticks collected in Wisconsin during the 1990s suggests that the EML pathogen had been established in Wisconsin for $\geq$2 decades (*17*).

In summary, our findings indicate that human infection caused by the EML pathogen continues to occur. This pathogen should be considered among the differential diagnoses when tickborne diseases are suspected among residents of Minnesota or Wisconsin or among persons with histories of travel to either state.

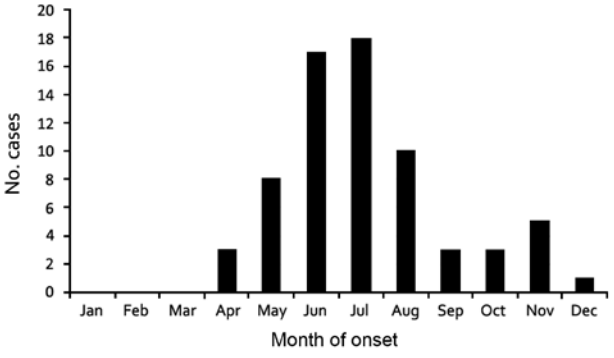


**Figure 2.** Month of symptom onset among 68 patients with *Ehrlichia muris*–like pathogen infection detected during 2007–2013, United States. Month of symptom onset was unknown for 1 patient.

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# Decreased Ebola Transmission after Rapid Response to Outbreaks in Remote Areas, Liberia, 2014

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We measured the reproduction number before and after interventions were implemented to reduce Ebola transmission in 9 outbreaks in Liberia during 2014. We evaluated risk factors for secondary cases and the association between patient admission to an Ebola treatment unit (ETU) and survival. The reproduction number declined 94% from 1.7 (95% CI 1.1–2.6) to 0.1 (95% CI 0.02–0.6) after interventions began. The risk for secondary infections was 90% lower for patients admitted to an ETU (risk ratio 0.1, 95% CI 0.04–0.3) than for those who died in the community. The case-fatality rate was 68% (95% CI 60–74), and ETU admission was associated with a 50% reduction in death (hazard ratio 0.5, 95% CI 0.4–0.8). Isolation and treatment of Ebola patients had the dual benefit of interrupting community transmission and improving survival.

The current Ebola virus disease (Ebola) epidemic in West Africa, caused by the Zaire strain, is the largest in history; >27,000 cases have been reported since Ebola was detected in Guinea in March 2014. Ebola in humans often begins with a nonspecific febrile illness and can progress to gastrointestinal symptoms, hemorrhage, sepsis, multiorgan failure, and death. Person-to-person transmission typically occurs through close contact with the blood or body fluids of a symptomatic infected person during care at home or in health care facilities or during traditional funeral rites (*1*). Case-fatality rates (CFRs) typically are high (68%–90%) for the Zaire strain (*2*,*3*). Although no cure exists for Ebola, supportive therapy, including intravenous fluids and electrolyte replacement, has been found to increase survival (*4*). Transmission of the virus can be reduced by isolation of patients, implementing infection control procedures while providing patient care, and avoiding direct contact with recently deceased persons (*1*).

The first case of Ebola in Liberia occurred in Lofa County in March 2014. The virus spread to the capital, Monrovia, by the end of May and to 10 of 15 counties by August 2014 (*5*). During July–December 2014, several Ebola outbreaks were detected in remote rural areas of Liberia, largely initiated by patients traveling from Monrovia (*6*). Because of difficult access, suboptimal medical care, limited telecommunications coverage, and low levels of health education in these areas, introduction of the Ebola virus led to several complex outbreaks requiring a rapid and coordinated public health response to halt transmission. Systematic prospective investigations of 9 of these outbreaks, all outside of Montserrado County, by the Ministry of Health and Social Welfare (MOHSW), the US Centers for Disease Control and Prevention, the World Health Organization (WHO), and other partners provided an opportunity to characterize Ebola transmission and measure the association among implementation of interventions, transmission, and survival.

## Methods

### Outbreak Investigations and Response

An Ebola outbreak was defined as ≥2 cases in a community within a 21-day period. Each remote community with an outbreak presented different challenges to an effective public health response, but as part of the interventions implemented in each outbreak, symptomatic persons were immediately isolated (through self-isolation in the home or transfer to an Ebola treatment unit [ETU]), and their contacts were identified and monitored. In 2 communities,

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challenges in accessibility combined with the presence of severely ill community members required the rapid establishment of temporary isolation and treatment facilities in the community. In the other communities, symptomatic residents were provided transport to an ETU after they traveled by foot to the nearest point accessible by an ambulance. Other interventions provided to the affected communities included promotion of Ebola prevention messages and training in safe and hygienic burials.

Standard MOHSW case investigation forms were completed for all case-patients through interviews with the case-patients or proxies. Case-patient status at the time of report was classified as alive or deceased. During the outbreak investigations, epidemiologists developed transmission chains retrospectively by identifying the source-patients for known cases and linking them through chains of infection to the index case. Prospectively, additional cases were identified through monitoring of contacts and active case finding (*7*). Cases were classified as suspected or probable on the basis of MOHSW guidelines, adapted from WHO-recommended case definitions (*7*). Confirmed cases were identified by laboratory diagnosis of Ebola using real-time reverse transcription PCR of a venous blood sample or a postmortem buccal swab.

During field investigations, the likely source-patient was identified for each case-patient through interviews with the case-patient or proxies; when multiple source-patients were possible (e.g., during intrahousehold transmission), the source-patient was considered missing for analysis purposes. The number of secondary cases generated by each case was determined from the transmission chain dendrograms when a clear epidemiologic link existed between a source-patient and ≥1 successive cases.

Missing information from case investigations was supplemented by manual searches of ETU and laboratory databases. Date of patient recovery was recorded from ETU databases and defined as the date of discharge; ETUs routinely discharged patients after symptoms had resolved and 1–3 blood samples tested negative for Ebola virus. For case-patients who survived their illness in the community without admission to an ETU, the date of recovery was the first date on which investigators could verify that the person was no longer symptomatic.

### Statistical Analysis

The minimum incubation period was calculated as the number of days between last exposure to the source-patient and symptom onset of the case-patient. The clinical serial interval was the number of days between dates of symptom onset of successive cases linked in a transmission chain.

We compared categorical variables using a $\chi^2$ test and changes in categorical variables over time using a Cochran-Armitage test for trend. Cases in each outbreak were classified as occurring before or after public health interventions began in the community based on date of symptom onset. The reproduction number $R$ (i.e., number of cases in an uninfected population that 1 case generates during its infectious period) was calculated as the mean number of secondary infections from cases that occurred before ($R_0$) and after ($R_t$) interventions began in the community. We computed 95% CIs for reproduction numbers by using a negative binomial model accounting for the extra correlation from data clustered by community. Percentage reduction in reproduction number was calculated as $(R_0–R_t)/R_0 \times 100\%$.

We calculated risk ratios (RRs) for infection of ≥1 secondary cases using generalized estimating equations with a log-binomial distribution (*8*). The extra correlation from clustering by community was accounted for by using an exchangeable correlation structure. RRs and 95% CIs were calculated and a Score $\chi^2$ test, adjusted for small sample sizes (*9*), of $p<0.05$ determined the statistical significance of variables (*10*). Percentage reduction in transmission was calculated as (1–RR) × 100%. We estimated survival distributions by case-patient admission to an ETU using a Kaplan-Meier curve accounting for the number of days between symptom onset and ETU admission and clustering by community. The association between admission to an ETU and survival was evaluated with a Cox proportional-hazards model by using the survival package in R v.3.1.1 (*11*). Admission to an ETU was a binary time-dependent variable entered into the model by using the counting process method. Survival was defined as the number of days from the date of symptom onset until death and was censored at the time of discharge from an ETU or recovery in the community. Robust SEs were used to calculate the 95% CI of the hazard ratio (HR) to account for correlated observations within communities. The assumption of proportional hazards was assessed with the use of Schoenfeld residuals. Percentage reduction in survival was calculated as (1–HR) × 100%.

### Ethical Considerations

This investigation was conducted as part of the Ebola public health response in Liberia. It was not considered to be human subjects research, in accordance with the US federal human subjects protection regulations and the US Centers for Disease Control and Prevention's Guidelines for Defining Public Health Research and Public Health Non-Research.

## Results

Fifteen outbreaks of Ebola occurred in remote areas of Liberia during July–December 2014 (*6*); 9 had transmission chains linking most cases to a source-patient and are included in this report (Figure 1; Table 1, http://wwwnc.cdc.gov/EID/article/21/10/15-0912-T1.htm). The time when

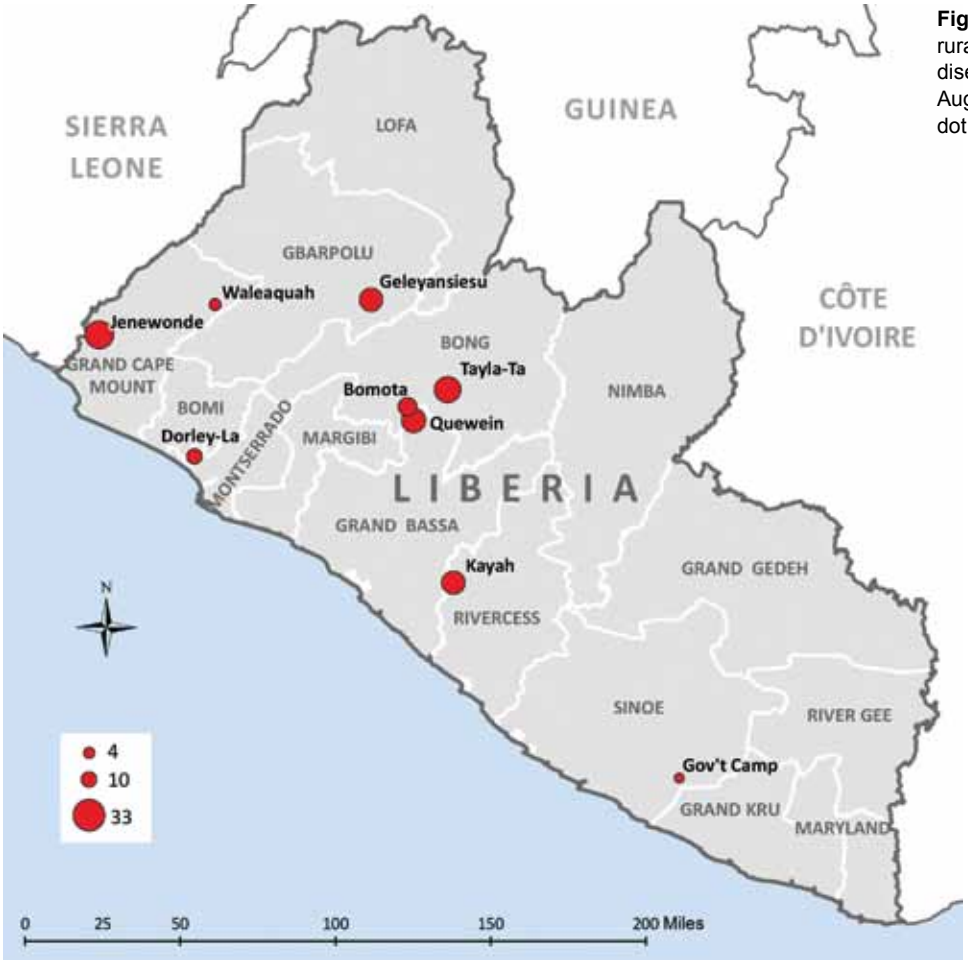


**Figure 1.** Communities in remote rural areas where Ebola virus disease outbreaks occurred, Liberia, August–December 2014. Size of red dot indicates number of cases.

MOHSW was notified of an outbreak to the first day of public health intervention in the community was a median of 32 days (range 9–58 days) (online Technical Appendix Figures 1, 2, http://wwwnc.cdc.gov/EID/article/21/10/15-0912-Techapp1.pdf).

### Description of Case-Patients

A total of 165 persons had an illness meeting the case definition for Ebola. Ninety-one (55%) patients were female, and median age was 33 years (range 15 days–84 years) for the 161 patients for whom age was known (Table 2). The most common symptoms reported during the case investigations were fever (92%), intense fatigue (86%), weight loss (63%), and muscle pain (58%) (Table 2).

Ebola was laboratory-confirmed in 115 (70%) case-patients, and 112 (68%) died; however, because 1 death resulted from drowning, the CFR was 68% (95% CI 60%–74%). One hundred (61%) case-patients were isolated and treated in an ETU. Of those admitted to an ETU, 51% (95% CI 41%–61%) died, compared with 94% (95% CI 85%–98%) of the 63 (38%) case-patients not admitted to an ETU ($p<0.0001$). Four (6%) case-patients (3 with laboratory-confirmed Ebola) were known to have survived their illness in the community without medical attention.

### Time Intervals

The median minimum incubation period was 8 days (mean 8.4 ± SD 3.7 days) (online Technical Appendix Figure 2, panel A), and the median clinical serial interval was 15 days (mean 15.1 ± SD 4.5 days) (online Technical Appendix Figure 2, panel B). Time intervals for patient outcomes and length of ETU stay can be found in online Technical Appendix Figures 3 and 4. The intervals between symptom onset of individual case-patients and the start of interventions in each community are presented in online Technical Appendix Figure 5.

### Secondary Cases

We identified the source-patient of 138 (90%) of the 155 nonindex cases. The number of secondary cases was determined for 157 (95%) case-patients. The proportion of cases for which the source-patient was identified did not differ before and after investigation (90% and 89%, respectively, $p = 0.89$). Most (76%) case-patients generated no secondary

**Table 2.** Characteristics of 165 case-patients with Ebola virus disease in 9 outbreaks in remote rural areas, Liberia, August–December 2014

| Characteristic | No. (%) patients* |
|---|---|
| Female sex | 91 (55) |
| Age, y† | |
| 0–14 | 33 (21) |
| 15–19 | 12 (7) |
| 20–29 | 25 (16) |
| 30–39 | 30 (19) |
| 40–49 | 26 (16) |
| 50–59 | 18 (11) |
| ≥60 | 17 (11) |
| Type of case | |
| Confirmed | 115 (70) |
| Probable | 38 (23) |
| Suspected | 12 (7) |
| Outcomes | |
| Admitted to Ebola treatment unit | 100 (61) |
| Died | 52 (51) |
| Recovered | 49 (49) |
| Not admitted to Ebola treatment unit | 64 (39) |
| Died | 59 (92) |
| Recovered | 4 (6) |
| Died accidentally | 1 (2) |
| Symptom | |
| Fever | 70 (92) |
| Intense fatigue | 64 (86) |
| Weight loss | 24 (63) |
| Muscle pain | 40 (58) |
| Headache | 31 (41) |
| Vomiting/nausea | 26 (37) |
| Difficulty breathing | 29 (39) |
| Abdominal pain | 27 (36) |
| Diarrhea | 26 (35) |
| Hiccups | 25 (34) |
| Difficulty swallowing | 7 (15) |
| Unexplained bleeding | 2 (3) |

*Percentages are proportions of non-missing data.
†Case-patients were a median of 33 years of age (range 15 days–84 years).

cases (median 0, mean 0.9, range 0–27) (Figure 2). Case-patients who died in the community generated 93% of the secondary cases, whereas case-patients admitted to an ETU generated 7%, and case-patients who survived their illness in the community generated <1%. Six case-patients, all of whom died in the community, infected 55% of the secondary case-patients identified.

The risk for secondary infections was lower for children <15 years of age (RR 0.2, 95% CI 0.1–0.5) and adults 40–49 (RR 0.2, 95% CI 0.1–0.5) than for adults ≥60 years of age, but age overall was not statistically associated with infection of secondary cases (p = 0.12) (Table 3). Compared with case-patients who died in the community, case-patients admitted to an ETU were associated with a 90% lower risk for infection of secondary cases (RR 0.1, 95% CI 0.04–0.3) (Table 3). Case-patients with symptom onset after interventions began in their community were significantly less likely to generate a secondary case than were case-patients who became ill before interventions started (RR 0.1, 95% CI 0.02–0.8).

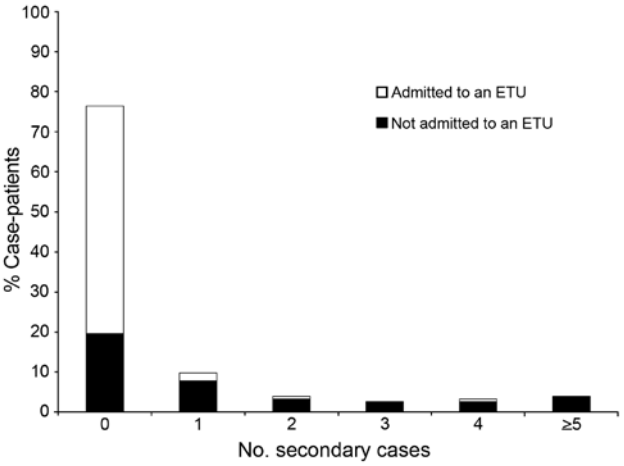


**Figure 2.** Distribution of Ebola virus disease case-patients by number of secondary cases generated and admission to an Ebola treatment unit (ETU) in remote rural areas of Liberia, August–December 2014.

During the preintervention period, the number of secondary cases ranged from 0 to 27, and the reproduction number was 1.7 (95% CI 1.2–2.6). After interventions began, the number of secondary cases was 0–4, and the reproduction number declined to 0.1 (95% CI 0.02–0.6) (Figure 3), a 94% decrease in transmission.

### CFRs Over Time and By Age

Overall CFR declined significantly over time (p = 0.002), from 92% in August and September to 60% in December (Figure 4). The CFR for case-patients admitted to an ETU was 67% in August and September and 50% in December (95% CI 22%–78%), but there was no

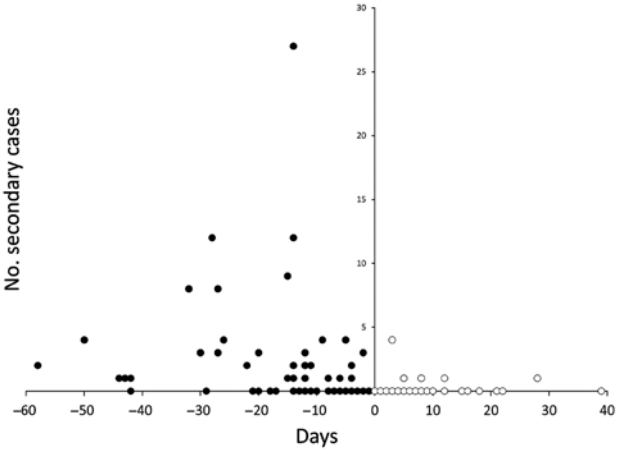


**Figure 3.** Number of Ebola virus disease secondary cases generated by case-patients, by time from symptom onset to start of interventions, in remote rural areas of Liberia, August–December 2014. Black circles indicate cases that occurred before the start of interventions (day 0); white circles indicate cases that occurred after interventions started.

**Table 3.** Risk factors for ≥1 secondary Ebola virus disease cases in outbreaks in remote rural areas, Liberia, August–December 2014

| Characteristic | ≥1 Secondary cases, no./total (%), n = 156 | Risk ratio (95% CI) | p value |
|---|---|---|---|
| Sex | | | 0.45 |
| F | 21/85 (25) | 1.2 (0.8–1.7) | |
| M | 16/71 (23) | Referent | |
| Age, y | | | 0.12 |
| 0–14 | 3/32 (9) | 0.2 (0.1–0.5) | |
| 15–19 | 3/8 (38) | 1.0 (0.4–2.5) | |
| 20–29 | 5/25 (20) | 0.6 (0.3–1.2) | |
| 30–39 | 8/30 (27) | 0.6 (0.3–1.0) | |
| 40–49 | 5/24 (21) | 0.2 (0.1–0.5) | |
| 50–59 | 5/17 (29) | 0.8 (0.4–1.5) | |
| ≥60 | 7/17 (41) | Referent | |
| Outcome | | | 0.02 |
| Admitted to Ebola treatment unit | 5/95 (5) | 0.1 (0.04–0.3) | |
| Recovered in the community | 1/4 (25) | 0.5 (0.1–3.3) | |
| Died in the community | 31/57 (54) | Referent | |
| Timing of case | | | 0.02 |
| Before intervention | 30/77 (39) | Referent | |
| After intervention | 5/75 (7) | 0.1 (0.02–0.8) | |

significant trend in CFR for case-patients admitted to an ETU (p = 0.38).

CFR by age group ranged from 88% for case-patients ≥60 years of age to 56% for those 50–59 years (Figure 5). Overall, however, CFR by age did not differ significantly by age group (p = 0.67).

### Survival Analysis

The Kaplan-Meier estimate of the median time to death from symptom onset for case-patients who reached an ETU was 11 days (95% CI 10–¥ days), compared with 8 days (95% CI 6–11 days) for case-patients not admitted to an ETU (Figure 6; Schoenfeld residuals in online Technical Appendix Figure 6). The Cox proportional-hazards model did not find the HR to vary by sex (p = 0.37) or age (p = 0.27), but admission to an ETU was associated with a 50% reduction in risk for death (HR 0.5, 95% CI 0.3–0.8, p = 0.04) (Table 4); this model was unadjusted because no other variables were found to be associated with survival in univariate modeling.

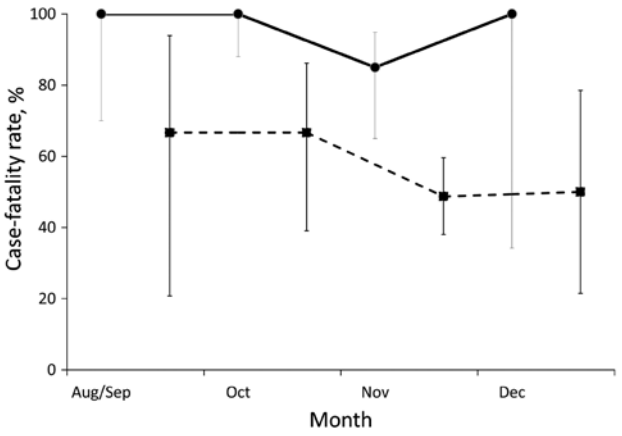


**Figure 4.** Case-fatality rates for Ebola virus disease, by case-patient admission to an Ebola treatment unit (ETU) and month of symptom onset, in remote rural areas of Liberia, August–December 2014. Dashed lines indicate case-patients admitted to ETU; solid lines indicate patients not admitted to ETU. Error bars indicate 95% CIs.

## Discussion

We found a 94% decrease in Ebola transmission after initiation of community interventions in 9 outbreaks in remote rural areas of Liberia during August–December 2014. Isolation and treatment of case-patients in an ETU was associated with a 90% lower risk for secondary cases than those who died in the community and with a 50% lower risk for death than those not admitted to an ETU. Liberia was declared free of Ebola on May 9, 2015 (*12*); however, 3 new cases were identified in July 2015 (*13*).

Although ETUs are a critical intervention to reduce Ebola transmission in the community (*14*), treatment of Ebola case-patients is limited to supportive care, for which

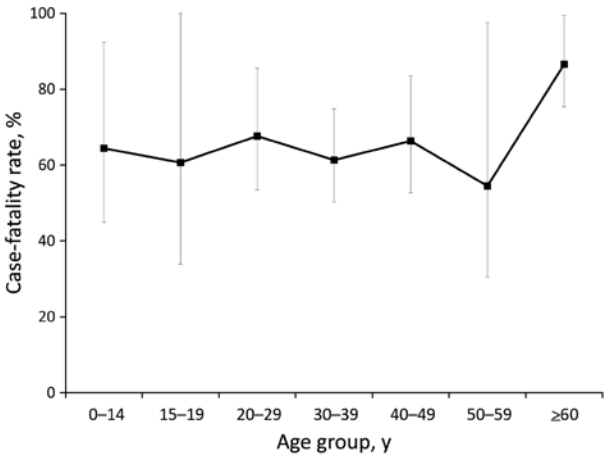


**Figure 5.** Case-fatality rates for Ebola virus disease, by case-patient age group, in remote rural areas of Liberia, August–December 2014. Error bars indicate 95% CIs.

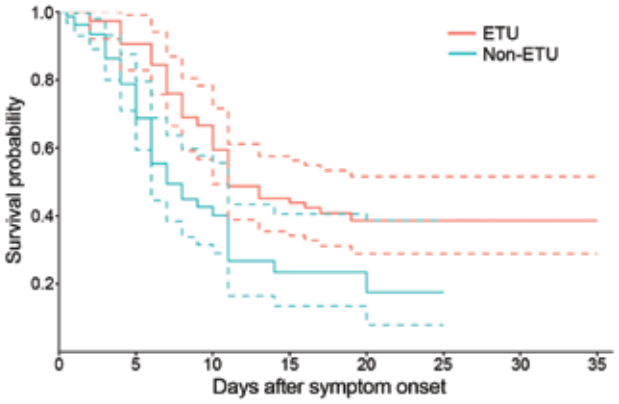


**Figure 6.** Kaplan-Meier survival curves comparing survival by admission to an Ebola treatment unit (ETU) in remote rural areas of Liberia, August–December 2014. Dashed lines indicate 95% CIs.

the efficacy and effectiveness remain unknown. The WHO Response Team found that the CFR was lower for hospitalized Ebola patients in West Africa (64%) than for all patients (71%) but recognized that this finding could result from multiple possible case ascertainment biases rather than a direct effect of ETU admission (*15*). The potential for bias also cannot be discounted for this study. If mild illness was less likely to be identified during community investigations or if severely ill patients were less likely to reach an ETU, our estimate of the impact of ETU admission on survival might be an overestimate. The data used in this analysis were collected prospectively by teams of experienced epidemiologists and local public health authorities. Complete transmission chains were developed, reducing the chances that cases were missed. In addition, robust statistical analyses adjusting for the number of days from symptom onset to ETU admission were used to account for the possibility that early treatment could increase the chances of survival or that a longer period before admission could introduce a survivor bias. With all the caveats inherent to an observational study, we believe that these data provide evidence that ETU admission improved the chances for patient survival in Liberia.

The reproduction number for Ebola after initiation of intervention declined significantly, from an average of 1.7 to 0.1 secondary cases infected. The reproduction number measured before the start of interventions was similar to that reported for Liberia during the early phase of the epidemic before most interventions, including isolation facilities at ETUs and safe burials, were widely available (*15*). With an average of only 0.1 secondary infections per case after the public health responses began, the outbreaks terminated rapidly. A study in Conakry, Guinea, that linked 152 Ebola cases in transmission chains found a significant decline in the reproduction number from 2.3 in March before the start of interventions to 0.7 for case-patients admitted to ETUs after interventions were implemented; the reproduction number for case-patients not admitted to ETUs did not decline significantly from the preintervention period, suggesting that ETUs were important in reducing transmission (*16*). We could not measure the impact of safe and hygienic burials on transmission, but increased admission of case-patients to ETUs clearly helped reduce Ebola transmission in the communities included in this report. However, this finding does not imply that we can attribute all transmission reduction in these outbreaks to outside intervention. Communities actively participated in the response and, in different times and places, took measures to protect themselves, including engaging in social distancing, washing hands, avoiding traditional burial practices, and sending patients for treatment outside the community. Although not measureable, community interventions most likely contributed to some of the decline in transmission reported during these outbreaks.

Infection of secondary cases was clustered among few persons; 6 source-patients, all of whom died in the community, accounted for more than half of the cases in this report. "Superspreader" events have been documented previously, although whether these case-patients had more contacts overall or more contacts during periods of higher viremia, such as during the terminal illness or after death (*1*,*16*), is not clear. Community deaths overall generated 93% of secondary infections in these outbreaks. In contrast to our results, attendance at funerals in urban Conakry accounted for only 6% of cases (*16*). Although Ebola transmission around death could be relatively more important in rural than in urban areas, the classification of funeral exposure used in the analysis by Faye et al. (*16*) is likely to have excluded many contacts around the terminal illness and preparation of the body for burial, which generally take place before the day of the funeral. Regardless, Ebola deaths in the community have the potential to cause substantial transmission, and illness and deaths associated with funeral attendance should be considered a critical trigger for investigations of possible Ebola transmission.

As in previous reports (*17*,*18*), the age distribution of case-patients in these outbreaks did not reflect the general population. In Liberia, children <15 years of age comprise 43% of the population (*19*) but accounted for only 21% of case-patients. This low percentage could be the result of underreporting of infections in children or variation in patterns by age of exposure, infection, and clinical manifestations. We believe that the intensive investigations of each outbreak in this report limited the likelihood that cases in children were missed, but we cannot exclude that possibility because children were more likely than adults to be buried secretly in Liberia (D. Allen, pers. comm.). During the Ebola outbreak in Kikwit, Zaire, in 1995, children were determined to be at lower risk for Ebola because they were

**Table 4**. Hazard ratios for survival during outbreaks of Ebola virus disease in remote rural areas, Liberia, August–December 2014

| Characteristic | Hazard ratio (95% CI) | p value |
|---|---|---|
| Sex | | 0.37 |
| F | 0.8 (0.5–1.3) | |
| M | Referent | |
| Age, y | | 0.27 |
| 0–14 | Referent | |
| 15–19 | 0.9 (0.2–4.2) | |
| 20–29 | 0.8 (0.4–1.8) | |
| 30–39 | 0.8 (0.4–1.7) | |
| 40–49 | 0.8 (0.3–1.8) | |
| 50–59 | 0.5 (0.2–1.9) | |
| ≥60 | 1.5 (0.7–3.4) | |
| Ebola treatment unit | | 0.04 |
| No | Referent | |
| Yes | 0.5 (0.3–0.8) | |

less likely to be exposed to body fluids (*20*). This lower risk also might be the case in Liberia, where children do not typically provide care to sick family members or participate in traditional funeral rites (*21*).

Although children appeared to be at lower risk than adults for Ebola, we did not find their CFR to be lower. In Sierra Leone, Schieffelin et al. found a significantly higher CFR for hospitalized persons ≥45 years (94%) than for those <21 years of age (57%) (*18*). In Guinea, Sierra Leone, and Liberia, an analysis of all reported cases found the odds for death to be higher for persons ≥45 years of age than for those <45 years (odds ratio [OR] 2.5, 95% CI 1.8–3.5), but the odds of dying for persons <15 years and ≥15 years of age did not differ (OR 1.2, 95% CI 0.8–1.7) (*15*). Although we found the highest CFR for case-patients ≥60 years of age, this CFR did not differ significantly from that of any other age group. This finding might be a limitation of our sample size or might indicate that the higher CFR for persons ≥45 years of age from other studies resulted from ascertainment bias arising from inclusion of only hospitalized case-patients or those reported to the surveillance system, whereas ascertainment of cases in this report was community-based.

Our dataset is subject to some important limitations. The primary objectives of the teams responding to the outbreaks were to facilitate patient care and interrupt Ebola transmission. The teams constructed transmission chains during outbreak responses primarily to identify any previously unrecognized case-patients or contacts who might continue to transmit Ebola in the community or spread it to other areas of the country; use of these data for epidemiologic analyses was a secondary priority. As a result, some data are missing, particularly critical dates, and there is most likely some inaccuracy in the data collected from proxies when the case-patient had died or left the area. These limitations are inherent to all datasets from this and similar epidemics when urgent response is the primary focus.

The data we present provide strong evidence that when capacity for isolation and treatment of Ebola is sufficient, rapid response strategies in remote areas that engage communities to promptly isolate and remove case-patients for care have the dual benefit of contributing to interruption of transmission and improving survival rates through treatment at ETUs. Provided basic interventions are implemented and communities are accepting, outbreaks of Ebola in rural areas can be controlled rapidly.

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# Possible Role of *Rickettsia felis* in Acute Febrile Illness among Children in Gabon

**Gaël Mourembou, Jean Bernard Lekana-Douki, Oleg Mediannikov, Sydney Maghendji Nzondo, Lady Charlene Kouna, Jean Claude Biteghe Bi Essone, Florence Fenollar, Didier Raoult**

*Rickettsia felis* has been reported to be a cause of fever in sub-Saharan Africa, but this association has been poorly evaluated in Gabon. We assessed the prevalence of this bacterium among children <15 years of age in 4 areas of Gabon; the locations were in urban, semiurban, and rural areas. DNA samples from 410 febrile children and 60 afebrile children were analyzed by quantitative PCR. Overall, the prevalence of *R. felis* among febrile and afebrile children was 10.2% (42/410 children) and 3.3% (2/60 children), respectively. Prevalence differed among febrile children living in areas that are urban (Franceville, 1.3% [1/77]), semiurban (Koulamoutou, 2.1% [3/141]), and rural (Lastourville, 11.2% [15/134]; Fougamou, 39.7% [23/58]). Furthermore, in a rural area (Fougamou), *R. felis* was significantly more prevalent in febrile (39.7% [23/58]) than afebrile children (5.0% [1/20]). Additional studies are needed to better understand the pathogenic role of *R. felis* in this part of the world.

Over the past decade, reported cases of malaria and associated deaths have declined in Africa (*1*). This decrease has led to a search for other causes of fever in Africa, where unexplained febrile illnesses are one of the major health problems. In some sub-Saharan Africa countries, malaria treatments are still administered without a biologic diagnosis. For example, an assessment of complicated malaria and other severe febrile illness cases in a pediatric ward in Libreville, Gabon, showed that 43.5% of the children who received an antimalarial treatment had microscopy test results negative for malaria (*2*).

Other studies have shown that, in addition to malaria, other bacterial infections are a major cause of fever in Africa (*3*–*6*). *Staphylococcus aureus*, *Streptococcus pneumoniae*, nontyphoidal *Salmonella* spp., *Klebsiella pneumoniae*, and *Escherichia coli* are the bacteria most often detected in sub-Saharan Africa by the culture method (*7*,*8*). The use of molecular tools has enabled the identification of the following fastidious bacteria as a cause of unexplained fevers in Africa: *Rickettsia* spp., including *R. felis* (*3*–*5*,*9*); *Coxiella burnetii* (*10*); *Tropheryma whipplei* (*11*); and *Borrelia* spp. (*12*,*13*). However, the epidemiology of many fastidious bacteria, such as *R. felis*, remains poorly understood. In rural areas of Senegal, the prevalence of *R. felis* was generally higher (7%–24%) than that in urban areas of sub-Saharan African, such as Franceville, Gabon (10%) (*14*).

*R. felis* is a gram-negative bacterium belonging to the spotted fever group of *Rickettsia* spp. In Gabon, the bacterium has been reported in arthropods, including *Ctenocephalides felis* cat fleas (*15*) and *Aedes albopictus* mosquitoes (*16*), and in humans (*14*). Similar to many African countries, Gabon has a strong disparity between health care in urban and rural areas; in rural areas, little is known about the epidemiology of infectious diseases. The aim of our study was to evaluate the prevalence of *R. felis* infection among febrile and afebrile children in rural and urban areas of Gabon and the possible role of *R. felis* in acute febrile illness.

## Materials and Methods

### Study Area

Gabon is a central African country located on the equator along the Atlantic Coast (Figure 1). The country has a low coastal plain and hilly inland areas and savannas to the east and south; 80% of Gabon is covered by forest. The tropical climate is hot and humid, and the seasons alternate in precipitation and length: short dry season, long rainy season, long dry season, short rainy season.

### Study Design and Participants

Patients were recruited at 4 health centers (Figure 1) located in 3 Gabon provinces. One center, the Regional Hospital Center Amissa Bongo of Franceville, is in an urban area of Haut-Ogooué Province. Two centers, the Regional Hospital Center Paul Moukambi of Koulamoutou and the Medical

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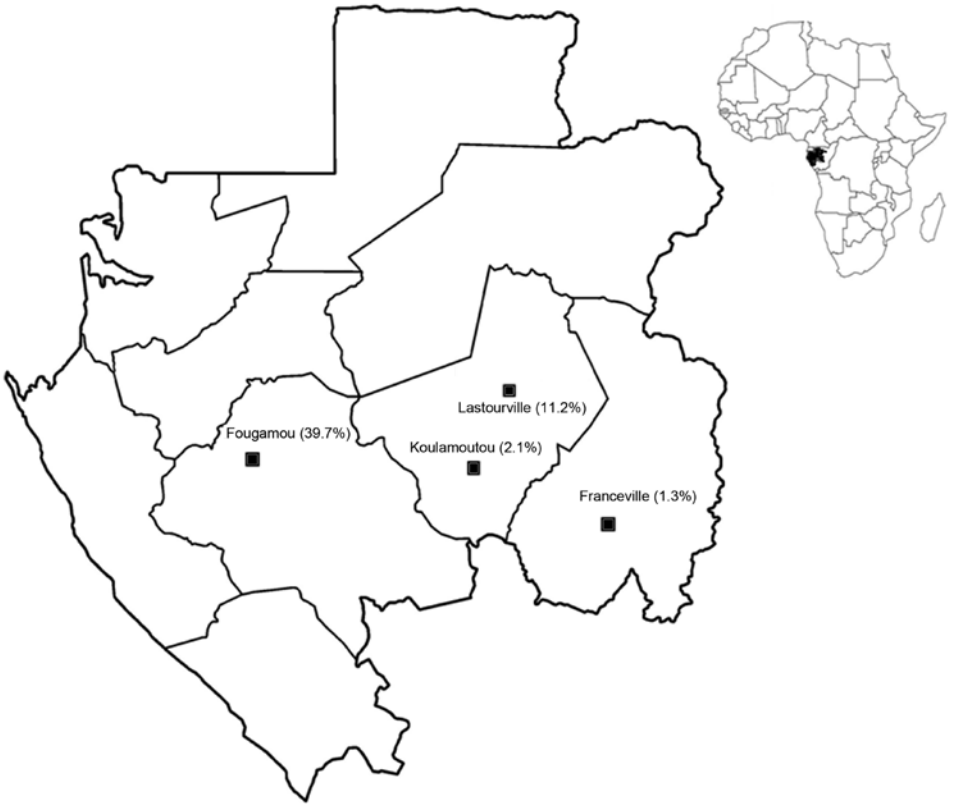


**Figure 1**. Four rural (Fougamou and Lastourville), semiurban (Koulamoutou), and urban (Franceville) locations in Gabon where children <15 years of age were tested for *Rickettsia felis* infection, April 2013–January 2014. Percentages in parentheses indicate the prevalence of *R. felis* infection among febrile children. Inset shows location of Gabon on the Atlantic Coast of Africa.

Center of Lastourville, are in semiurban and rural areas, respectively, of Ogooué Lolo Province. The fourth center, the Medical Research Unit of Ngounie in Fougamou, is in a rural area of Ngounié Province.

The National Ethics Committee of Gabon approved this prospective study (no. 0023/2013/SG/CNE). Written informed consent forms and questionnaires were completed by parents or legal guardians upon a child's enrollment in the study.

During April 2013–January 2014, a total of 525 children <15 years of age were recruited for the study; 465 of the children were febrile (axillary temperature >37.5°C), and 60 were afebrile (controls). Febrile children were recruited from the pediatric outpatient clinics at the 4 health care centers. The control group was recruited from children who had accompanied their sick parents to the health care centers. Children in the control group had to be free of fever for at least 1 week before study inclusion.

### Sample Collection and Molecular Analysis

Molecular analyses were performed on DNA extracts from blood samples from each child; blood smears, serologic testing, and culture were not done. After a child's parent or legal guardian was interviewed, a blood sample was collected into an EDTA tube. World Health Organization guidelines for blood collection were followed, including guidelines for hand hygiene, use of sterile tubes, and skin disinfection with 70% alcohol. The International Center of Medical Research of Franceville, which has a well-trained staff with expertise in infectious diseases, performed DNA extraction by using the E.Z.N.A. Blood DNA Maxi Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocol (*17*). A total of 150 μL of DNA extract was obtained from each sample. The extracts were stored at –20°C before being sent on ice to URMITE (Unité de Recherche sur les Maladies Infectieuses et Tropicales, Marseille, France) for molecular analyses.

Specific quantitative PCR (qPCR) was performed by using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Marnes-la-Coquette, France). qPCR Master Mix (Eurogentec, Liege, Belgium) was prepared according to the manufacturer's instructions; for each reaction, 15 μL of Master Mix was added to 5 μL of DNA. The quality of extracted DNA and the lack of PCR inhibitors were systematically checked by targeting a housekeeping gene, human β-actin (*14*). Positive (*R. felis* DNA) and negative (mix alone) controls were also systematically used for each PCR assay. All samples were screened by *Rickettsia* spp.–specific qPCR targeting the *gltA* gene and by *R. felis*–specific qPCR targeting the *bioB*, *orfB*, and *vapB1* genes (*14*) (Table 1). Samples positive for at least 2 genes were considered positive.

**Table 1**. Primers and probes used in *Rickettsia* spp.–specific *and R. felis*–specific quantitative qPCR screening of blood samples from children <15 years of age in Gabon*

| Pathogens, target gene | Primers, forward; reverse 5'→3' | Probe, 5'→3' |
|---|---|---|
| *Rickettsia* spp | | |
| RKND03 (*gltA*) | GTGAATGAAAGATTACACTATTTAT<br>GTATCTTAGCAATCATTCTAATAGC | 6FAM-CTATTATGCTTGCGGCTGTCGGTTC-TAMRA |
| *R. felis* | | |
| *bioB* (*0527*) | ATGTTCGGGCTTCCGGTATG<br>CCGATTCAGCAGGTTCTTCAA | 6FAM-GCTGCGGCGGTATTTTAGGAATGGG-TAMRA |
| *orfB* | CCCTTTTCGTAACGCTTTGCT<br>GGGCTAAACCAGGGAAACCT | 6FAM-TGTTCCGGTTTTAACGGCAGATACCCA-TAMRA |
| *vapB1* | TGTCTTTCATGAATTGATCAGCA<br>AGGCGAAAGCTTTGACGTG | 6FAM-AAGGCTTGGTTTCTGCGGGC-TAMRA |

*The origin of the quantitative PCR is described in (*14*).

### Statistical Analysis

Data were analyzed by using Epi Info software version 7.0.8.0 (Centers for Disease Control and Prevention, Atlanta, GA, USA). Mantel-Haenszel $\chi^2$ and Fisher exact tests were used to compare the prevalence between groups (i.e., febrile and afebrile children), seasons, and geographic areas and by the participants' sex and age. A 2-tailed p value <0.05 was considered statistically significant.

## Results

### Recruitment

A total of 465 febrile children were recruited from Franceville (n = 80), Koulamoutou (n = 167), Lastourville (n = 155), and Fougamou (n = 63) (Table 2). However, 55 of these children were excluded from the statistical analysis because their sex and age data were unavailable; 3 of the excluded children were from Franceville, 26 from Koulamoutou, 21 from Lastourville, and 2 from Fougamou. Of the 410 children included in the statistical analysis, 52.7% (216/410) were recruited during a rainy season, and 47.3% (194/410) were recruited during a dry season. A total of 60 afebrile children were recruited and enrolled in parallel from Franceville (n = 24), Fougamou (n = 20), and Lastourville (n = 16); no afebrile children were enrolled from Koulamoutou. The 410 febrile patients included in the statistical analysis consisted of 212 boys and 198 girls (sex ratio 1.07).

### *R. felis* in Febrile and Afebrile Children in Gabon

*R. felis* DNA was detected in 42 (10.2%) of 410 analyzed samples from febrile children (Table 3). The bacterium was detected significantly more frequently during the rainy season (15.3% [33/216 samples]) than the dry season (4.6% [9/194 samples]; p<0.001). The prevalence among boys (10.8% [23/212]) and girls (9.6% [19/198]) did not differ significantly (p = 0.74). Among febrile children, *R. felis* prevalence varied by age group: 8.5% (11/129 children) among children 0–1 year of age, 15.2% (16/105) among children >1– 3 years of age, 11.5% (10/87) children >3–5 years of age, 7.7% (3/39) among children >5–7 years of age, 6.7% (2/30) among children >7–9 years of age, and 0 (0/20) among children >9–15 years of age (Figure 2, panel A). The prevalence of *R. felis* among febrile children did not differ substantially by age, but the prevalence did increase progressively from Franceville (1.3% [1/77]) to Koulamoutou (2.1% [3/141]) to Lastourville (11.2% [15/134]) to Fougamou (39.7% [23/58]); however, no adjustments were made when testing these pairs (Figure 2, panel B). The prevalence was statistically lower in Franceville than in Lastourville (odds ratio [OR] 0.1, 95% CI $2.5 \times 10^{-3}$–0.7; p = 0.006), in Franceville than in Fougamou (OR 0.02, 95% CI $5 \times 10^{-4}$–0.1, p<0.001), in Koulamoutou than in Lastourville (OR 0.17, 95% CI 0.03–0.63; p = 0.002), in Koulamoutou than in Fougamou (OR 0.03, 95% CI $6 \times 10^{-3}$–0.12; p<0.001), and in Lastourville than in Fougamou (OR 0.19, 95% CI 0.08–0.43; p<0.001).

**Table 2.** *Rickettsia felis* test results and demographic data for children recruited for sampling in Gabon, April 2013–January 2014*

| Site | Population | No. *R. felis* positive/no. tested (%) | | Latitude, longitude | Altitude, m | Lifestyle | Vegetation |
|---|---|---|---|---|---|---|---|
| | | Febrile children | Afebrile children | | | | |
| Franceville | ≈56,000 | 1/77 (1.3) | 1/24 (4.2) | 1°37′14.52″S, 13°34′57.72″E | 333 | Urban | Savannah |
| Koulamoutou | ≈17,000 | 3/141 (2.1) | None enrolled | 1°8′20.65″S, 12°28′0.2″E | 349 | Semiurban | Plantations and degraded forest |
| Lastourville | ≈10,000 | 15/134 (11.2) | 0/16 | 0°49′1.2″S, 12°42′0″E | 483 | Rural | Rainforest |
| Fougamou | ≈4,100 | 23/58 (39.7) | 1/20 (5.0) | 1°13′0.01″S, 10°36′0″E | 108 | Rural | Plantations and degraded forest |

*For all sites, the short dry season occurred during mid-December–mid-February, the short rainy season occurred during mid-February–mid-May, the long dry season occurred during mid-May–mid-September, and the long rainy season occurred during mid-September to mid-December.

**Table 3.** Prevalence of *Rickettsia felis* infection among febrile and afebrile children <15 years of age, Gabon, April 2013–January 2014*

| Location, participants' fever status | No. positive children/no. tested (%), by age, y | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0–1 | >1–3 | >3–5 | >5–7 | >7–9 | >9–15 | All ages |
| Fougamou | | | | | | | |
| Febrile | 7/22 (31.8) | 8/11 (72.7) | 4/11 (36.4) | 2/6 (33.3) | 2/5 (40.0) | 0/3 | 23/58 (39.7) |
| Afebrile | 1/5 (20.0) | 0/4 | 0/3 | 0/3 | 0/2 | 0/3 | 1/20 (5.0) |
| Lastourville | | | | | | | |
| Febrile | 4/38 (10.5) | 7/42 (16.7) | 3/34 (8.8) | 1/9 (11.1) | 0/8 | 0/3 | 15/134 (11.2) |
| Afebrile | 0/2 | 0/6 | 0/5 | 0/1 | 0/2 | NA | 0/16 |
| Koulamoutou† | | | | | | | |
| Febrile | 0/55 | 0/35 | 3/24 (12.5) | 0/14 | 0/7 | 0/6 | 3/141 (2.1) |
| Franceville | | | | | | | |
| Febrile | 0/14 | 1 /17 (5.8) | 0/18 | 0/10 | 0/10 | 0/8 | 1/77 (1.3) |
| Afebrile | 0/10 | 1/7 (14.2) | 0/1 | 0/4 | 0/2 | NA | 1/24 (4.2) |
| Total cases | 12/146 (8.2) | 17/122 (13.9) | 10/96 (10.4) | 3/47 (4.1) | 2/36 (5.5) | 0/23 | 44/470 (9.4) |

*NA, not available.
†No afebrile children in Koulamoutou were enrolled in the study.

Overall, the prevalence of *R. felis* among febrile children was significantly higher in the rural areas (Lastourville and Fougamou; 19.8% [38/192 children]) than in the urban area (Franceville; 1.3% [1/77 children]; p<0.001). Among the 60 afebrile children, only 2 (3.3%; both girls) were positive for *R. felis*; the girls were 1 and 3 years of age and were from Fougamou and Franceville, respectively. In all, *R. felis* DNA was detected in 10.2% (42/410) of febrile children and in 3.3% (2/60) of afebrile children; this difference was not significant (p = 0.09).

### *R. felis* at Each Sampling Location

#### Fougamou, Ngounié Province

*R. felis* DNA was detected in 23 (39.7%) of 58 febrile children at this rural location (Table 3; Figure 2). Prevalence during the rainy season (48.7% [19/39 children]) was higher than that during the dry season (21.1% [4/19 children]; p = 0.05). *R. felis* prevalence among boys (50% [15/30]) and girls (28.6% [8/28]) did not differ significantly (p = 0.11). *R. felis* prevalence also did not differ significantly by age, even though prevalence was higher among 1- to 3-year-old children (Table 3). Overall, *R. felis* DNA was detected significantly more frequently in febrile (39.7% [23/58]) than afebrile (5.0% [1/20]) children (p = 0.004).

#### Lastourville, Ogooué Lolo Province

*R. felis* DNA was detected in 15 (11.2%) of 134 febrile children in this rural location (Table 3; Figure 2). *R. felis* prevalence during the rainy season (15.8% [12/76 children]) was higher than that during the dry season (5.2% [3/58 children]), but the difference was not statistically significant (p = 0.05). Prevalence among boys (7.6% [6/79]) and girls (16.4% [9/55]) did not differ significantly (p = 0.17). Prevalence also did not differ significantly by age (Table 3). *R. felis* DNA was detected in 15 (11.2%) of 134 febrile children and in 0 of 16 afebrile children; this difference was not statistically significant (p = 0.3).

#### Koulamoutou, Ogooué Lolo Province

*R. felis* DNA was detected in 3 (2.1%) of 141 febrile children in this semiurban location (Table 3; Figure 2). Prevalence during the rainy season (2.9% [2/68 children]) was higher than that during the dry season (1.4 [1/73 children]; p = 0.6). *R. felis* DNA was detected in 1 (1.6%) of 63 boys and in 2 (2.6%) of 78 girls (p≤1); 2 of these children were 4 years of age, and 1 was 5 years of age. As stated above, no afebrile children were enrolled from Koulamoutou.

#### Franceville, Haut-Ogooué Province

*R. felis* DNA was detected in 1 (1.3%) of 77 febrile children and in 1 (4.2%) of 24 afebrile children (p = 0.3) in this urban area (Table 3; Figure 2). The infected febrile child was a 2-year-old boy, and the infected afebrile child was a 3-year-old girl; both children became infected during the dry season.

## Discussion

The lack of molecular tools in many health centers in countries in sub-Saharan Africa limits the management of all febrile illnesses in these areas. In this study, we used molecular tools (PCR assays) to assess the prevalence of *R. felis* in blood specimens from febrile and afebrile children from rural, semiurban, and urban areas of Gabon. One of the most frequent pitfalls of PCR assays is the contamination of samples, which can occur any time during or after collection of the samples, including during their use in the laboratory. Over the years, the URMITE laboratory has developed strategies and applied rigorous procedures to prevent and detect contamination (*18*–*21*). For example, we require that 2 different PCR assays show positive results before we conclude that a sample is positive (*21*).

In this study, we used a *Rickettsia* spp.–specific qPCR targeting the highly conservative *gltA* gene and an *R. felis*–specific qPCR targeting the *bioB*, *orfB*, and v*apB1* genes. The *Rickettsia* spp.–specific assay can amplify almost all *Rickettsia* spp., including *R. conorii* and

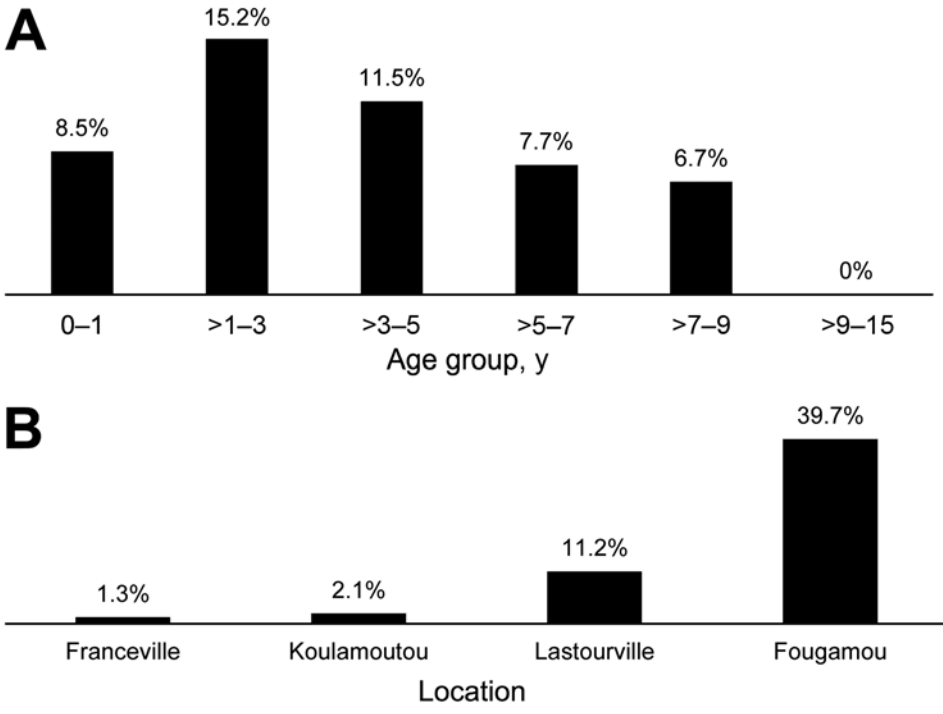


**Figure 2**. Prevalence of *Rickettsia felis* infection among febrile children <15 years of age in Gabon, April 2013–January 2014. A) Prevalence by age group. B) Prevalence by location.

*R. rickettsii*, but it is less sensitive than the *R. felis*–specific assay. When a sample in our study was positive by the *Rickettsia* spp.–specific qPCR, it was also positive by the *R. felis* qPCR. Several samples had positive results for only 2 of the 3 genes targeted by the *R. felis*–specific qPCR. This discrepancy might be explained by possible genetic diversity of the *R. felis* strains. The 3 genes used for the *R. felis*–specific assay are not all conservative, so it is possible that some strains have a certain degree of genetic diversity that may occasionally cause false-negative PCR results. Our results were also systematically validated by using rigorous criteria. To check the quality of each PCR run, we used negative controls (i.e., PCR mix without template) with every tenth sample, and we used 2 positive controls (*R. felis* DNA) per run. In addition, we required that all negative and positive controls be systematically correct (i.e., negative and positive, respectively) before validating each PCR run, and a sample was not considered *R. felis*–positive unless confirmed by at least 2 of the 4 sequences of targeted DNA. Thus, we consider our results to be valid. The fact that *R. felis* DNA has not been detected in samples (from febrile patients in France and Tunisia) previously analyzed in our laboratory (*14*) provides further support of our team's ability to prevent and detect contamination during PCR runs. Furthermore, it has been reported that the prevalence of *R. felis* is low in northern Africa countries (France, Algeria, Morocco, Tunisia) and increases in southern Africa countries (Mali, Senegal, Gabon) (*14*).

Consistent with our previous findings from Franceville in Haut-Ogooué Province (*14*), our findings from this study confirmed the presence of *R. felis* bacteremia in febrile children in Gabon and showed that the prevalence of infection was higher in rural than urban and semiurban areas. This fastidious bacterium was previously found in arthropods in Franceville (*15*), including the cat flea, *C. felis*. The bacterium was also detected in *A. albopictus* mosquitoes in Libreville in Estuaire Province (*16*). Data from our study also confirm the presence of *R. felis* in children in Ogooué lolo and Ngounié Provinces. Therefore, *R. felis* is widespread in Gabon, and its prevalence should be assessed in other areas of the country.

Common microorganisms involved in bacteremia (*S. aureus*, *Streptococcus pyogenes*, *E. coli*, *K. pneumoniae*, *Salmonella* spp., and *S. pneumoniae*) were previously assessed in Gabon by using standard culture methods (*8*), but the prevalence of fastidious bacteria, which are mainly detected by using molecular techniques, was not studied. There is a need to include these sensitive methods in diagnostic determinations. Our findings, plus those from studies in Senegal (*14*,*22*), Mali (*14*), Kenya (*5*), Ethiopia (*23*,*24*), Cameroon (*25*), Democratic Republic of the Congo (*26*,*27*), Ivory Coast (Côte d'Ivoire) (*6*), and Zimbabwe (*28*), show that *R. felis* is widespread in sub-Saharan Africa countries (*29*). However, its prevalence changes according to the season, year, area (rural, urban, and semiurban), country, and age of those infected.

A comparison of our findings with previously reported data showed that *R. felis* prevalence among febrile children

in Franceville decreased from 10% in 2012 (*14*) to 1.3% in 2014. In addition, the variation in the *R. felis* prevalence between urban (1.3%) and rural (39.7%) areas of Gabon showed that *R. felis* is unequally distributed in the country. Differences in the prevalence of a possible vector or reservoir, or both, and disparate health care–associated conditions, including environmental conditions, poverty, and the availability and quality of health care facilities, between rural and urban areas may influence the distribution of *R. felis* in Gabon; however, these factors have not been determined. An increased prevalence of *R. felis* was observed during the rainy season. This same finding was described in Senegal, where the prevalence of infection in the rural areas was 24 times higher than that in urban areas of Algeria (*14*). Together, the finding suggests that *R. felis* is more prevalent in rural areas and during the rainy season in sub-Saharan Africa.

*R. felis* was previously found in *C. felis* fleas from a pet monkey in Gabon, but the data concerned only 1 region, Franceville (*15*). *R. felis* has been reported to be absent from *C. felis* fleas in rural areas of Senegal, where *R. felis* is common (*30*). The factors explaining the spread of *R. felis* in Gabon should be evaluated in further studies. Although *R. felis* is widespread in Africa and unequally distributed in Gabon and Senegal (*14*), its prevalence varies by country: 15.0% in Senegal (*14*), 3.0% in Mali (*14*), and 7.2% in Kenya (*5*). In our study, *R. felis* was mainly detected in young febrile children <5 years of age (primarily in those 1–3 years of age). In a rural area of Senegal (Dielmo and Ndiop), the incidence of *R. felis* infection has also been reported to be higher (reaching 36%) among febrile children 1–3 years of age, but the incidence was lower (0.1%) in persons >15 years of age (*14*). It has been reported that the seroprevalence of *R. felis* increases with age in areas where the bacterium is endemic (*5*). This increase is probably due to exposure to the bacterium during the course of a lifetime, leading to protection by a progressive development of immunity against this bacterium in adults.

Most of the fever-associated studies conducted in Africa failed to use a control group of afebrile persons. Consequently, when a pathogen was detected in febrile patients, it was systematically and automatically considered as the cause of fever (*7*). In some cases, we have also observed a mistake in methodology: data comparisons were performed between samples from afebrile persons in an occidental area and from febrile persons in Africa (*31*). The epidemiology of microorganisms depends on the studied areas, and the positive predictive value of a disease depends on its symptoms and epidemiology. For example, *Plasmodium falciparum*, the primary agent of malaria, is commonly detected in blood specimens from apparently healthy, afebrile persons in Sub-Saharan Africa; prevalence can reach 20% in Ethiopia and 32% in Senegal (*32,33*). Respiratory viruses, including influenza virus, have also been found in 12% of nasopharyngeal samples of asymptomatic Hajj pilgrims (*34*). More recently in Tanzania, the prevalence of *S. pneumoniae* DNA was less frequently detected in febrile (5.1%) than afebrile (6.3%) persons (*35*). Thus, these examples show that even well-known pathogens may be detected in blood or respiratory secretions of afebrile persons. The inclusion of control groups of participants in studies is indispensable to a better understanding of infectious diseases; the use of controls has shown the existence of carriers of well-known pathogens, emphasizing that it is not easy to interpret data about the potential pathogenic role of a microorganism. The finding of *R. felis* in febrile versus afebrile persons is not well characterized. The overall prevalence of *R. felis* in Gabon was higher in febrile (10.2%) than afebrile (3.3%) children, but the difference was not statistically significant ($p = 0.09$). Of more interest, in rural Fougamou, the difference in prevalence was significantly higher in similarly aged children with and without fever (39.7% vs. 5.0%, respectively; $p = 0.004$). Therefore, the presence of *R. felis* in febrile and afebrile persons should not exclude that this bacterium is a cause of fever in sub-Saharan Africa.

In 2010, independent research teams detected *R. felis* in blood specimens from febrile patients in 2 different areas of Africa (eastern and western) (*3*,*4*). In other studies, these teams confirmed and extended the preliminary data: 1 team showed that the presence of *R. felis* was 2.2 times higher in blood specimens from febrile persons compared with afebrile persons in Kenya (*5*), and the other team showed that the prevalence of *R. felis* was significantly higher in febrile (15.0%) than afebrile (4.0%) persons in Senegal (*14*). In Senegal, an 8-month-old febrile girl was cured of *R. felis* infection after treatment with doxycycline (*36*). The presence of *R. felis* has also been observed in blood specimens from febrile patients in Asia (*37*). The higher prevalence of *R. felis* among febrile persons compared with healthy persons in our study led us to suspect that this microorganism plays the role of pathogen. However, the presence of the microorganism may be a cofactor or the cause of a previous event not yet determined. Another hypothesis would be that blood specimens may be contaminated by surface bacteria, including *R. felis*, which has been detected on the skin of healthy persons in Senegal (*38*).

In summary, the *R. felis* bacterium is widespread in Gabon, but it primarily occurs in rural areas and is most prominent during the rainy season. *R. felis* is also more prevalent among febrile than afebrile children in rural areas of Gabon. More studies will help to better understand the pathogenic role of *R. felis* in this part of the world.

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Mr. Mourembou, a PhD student in infectious diseases, works in the Unit of Research on Emergent Infectious and Tropical Diseases in Marseille, France. His main research interest is fever of unknown origin in Gabon.

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# Utility of Oral Swab Sampling for Ebola Virus Detection in Guinea Pig Model

**Jessica R. Spengler, Ayan K. Chakrabarti, JoAnn D. Coleman-McCray, Brock E. Martin, Stuart T. Nichol, Christina F. Spiropoulou, Brian H. Bird**

To determine the utility of oral swabs for diagnosing infection with Ebola virus, we used a guinea pig model and obtained daily antemortem and postmortem swab samples. According to quantitative reverse transcription PCR analysis, the diagnostic value was poor for antemortem swab samples but excellent for postmortem samples.

Ebola virus (EBOV) causes Ebola virus disease (EVD), which results in a high number of deaths in humans. EBOV is the etiologic agent of the ongoing EVD outbreak in West Africa. Nonadapted EBOV causes disease in nonhuman primates, but adaptation is required for the virus to cause disease in rodent models (*1–4*). Fatal disease has been observed in 20% of guinea pigs infected with wild-type (WT) nonadapted EBOV, but a uniformly lethal guinea pig–adapted EBOV isolate was found to have developed after a limited number of serial infection passages in guinea pigs (*3,5,6*).

Real-time quantitative reverse transcription PCR (qRT-PCR) is used to detect EBOV in the current West Africa outbreak. Appropriate sample collection and knowledge of interpreting results on the basis of specimen type are essential for accurate triage of patients thought to have EVD. Oral swab sampling for postmortem EBOV diagnosis has been supported by use of a nonhuman primate model (*7*), and oral swab sampling for antemortem EVD diagnosis has been a major consideration in the current outbreak because collection of swab samples is less invasive than collection of serum samples and poses a much lower risk of transmitting EBOV to the person obtaining the sample than traditional phlebotomy. However, the utility of oral swabs for antemortem testing has not been investigated in detail under controlled experimental conditions. In addition, Bausch et al. have suggested that the oral milieu, such as saliva composition and oral cavity tissue structure, may potentially inhibit diagnostic capabilities of oral swab sampling (*8*).

Wong et al. have shown that oral swabbing can be used to detect virus and shedding in guinea pigs at isolated intervals after infection (*9*). We investigated oral swab sampling as an antemortem means of diagnosing EVD and used qRT-PCR to detect EBOV RNA in daily oral swab samples obtained from guinea pigs infected with guinea pig–adapted EBOV (GP-EBOV) and with WT-EBOV.

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## The Study

Procedures and experiments described herein were approved by the Centers for Disease Control and Prevention (CDC) Institutional Animal Care and Use Committee and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals (*10*). CDC is a fully accredited research facility of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Healthy adult male and female strain 13/N guinea pigs, 1.0–2.5 years of age, were housed in a Biosafety Level 4 laboratory in microisolator cage systems filtered with high-efficiency particulate arrestance filters. Groups of 5 animals, distributed proportionally by age and sex, were inoculated intraperitoneally with a 50% tissue culture infectious dose ($TCID_{50}$) at low (5 $TCID_{50}$) or high (5,000 $TCID_{50}$) levels of GP-EBOV-Mayinga, or with $5 \times 10^5$ $TCID_{50}$ of either the WT-EBOV-Mayinga 1976 variant (Ebola virus/*H. sapiens*-tc/COD/1976/Yambuku-Mayinga) or the WT-EBOV-Makona 2014 variant (Ebola virus/*H. sapiens*-tc/LBR/2014/Makona; GenBank accession no. KP178538). To serve as negative controls, 3 animals were inoculated intraperitoneally with Dulbecco's Modified Eagle's Medium. Animals were monitored for signs of clinical illness, and body weight and temperature readings were obtained daily. Oral swab samples were collected daily for isolation of RNA and analyzed by qRT-PCR. Postmortem oral swab samples were obtained from 10 animals that were euthanized because of severe clinical illness consistent with EBOV. Carcasses of the dead animals were kept in an incubator at 30°C to simulate conditions in equatorial Africa. Samples were obtained from 9 of the 10 animals for up to 5 days after death and from 1 animal at 2 days after death. In addition to oral swab samples, paired blood samples were collected from the cranial vena cava of anesthetized animals at 3 days postinfection (dpi) and by cardiac puncture at the time of death for euthanized animals.

Low and high doses of GP-EBOV-Mayinga were uniformly lethal. Clinical illness was delayed in 1 animal in the high-dose group; the animal was euthanized at 12 dpi, but all other animals were euthanized by 9 dpi. One animal infected with nonadapted WT-EBOV-Mayinga was euthanized

at 9 dpi because of clinical illness. No severe clinical illness developed in any of the other animals infected with WT-EBOV-Mayinga or WT-EBOV-Makona (Figure, panel A). Fever developed in all animals infected with low- and high-dose GP-EBOV-Mayinga, in 20% of animals infected with WT-EBOV-Makona or WT-EBOV-Mayinga, and in none of the negative control animals. Hypothermia, typical during the terminal phases of many disease processes, was observed in animals with end-stage EVD (Figure, panel B). Substantial weight loss (>15%) was observed in all febrile animals (Figure, panel C). The 1 animal infected with WT-EBOV-Makona that showed clinical signs experienced transient fever and weight loss but started to regain weight by 9 dpi.

Oral swab samples were analyzed by qRT-PCR targeting the EBOV nucleoprotein gene; 18s ribosomal RNA levels were also analyzed to serve as a sampling control. EBOV RNA abundance was calculated by comparing the cycle threshold values to an in vitro–transcribed small-segment RNA standard of known copy number. All oral swab samples that were collected 0–4 dpi were negative for EBOV nucleoprotein RNA (Table). At 3 dpi, blood samples from 7 (41%) of 17 infected animals from which blood samples could be obtained were positive for EBOV, but no viral RNA was detected in any of the paired oral swab samples. The earliest detection of EBOV RNA by oral swabbing was at 5 dpi in an animal infected with

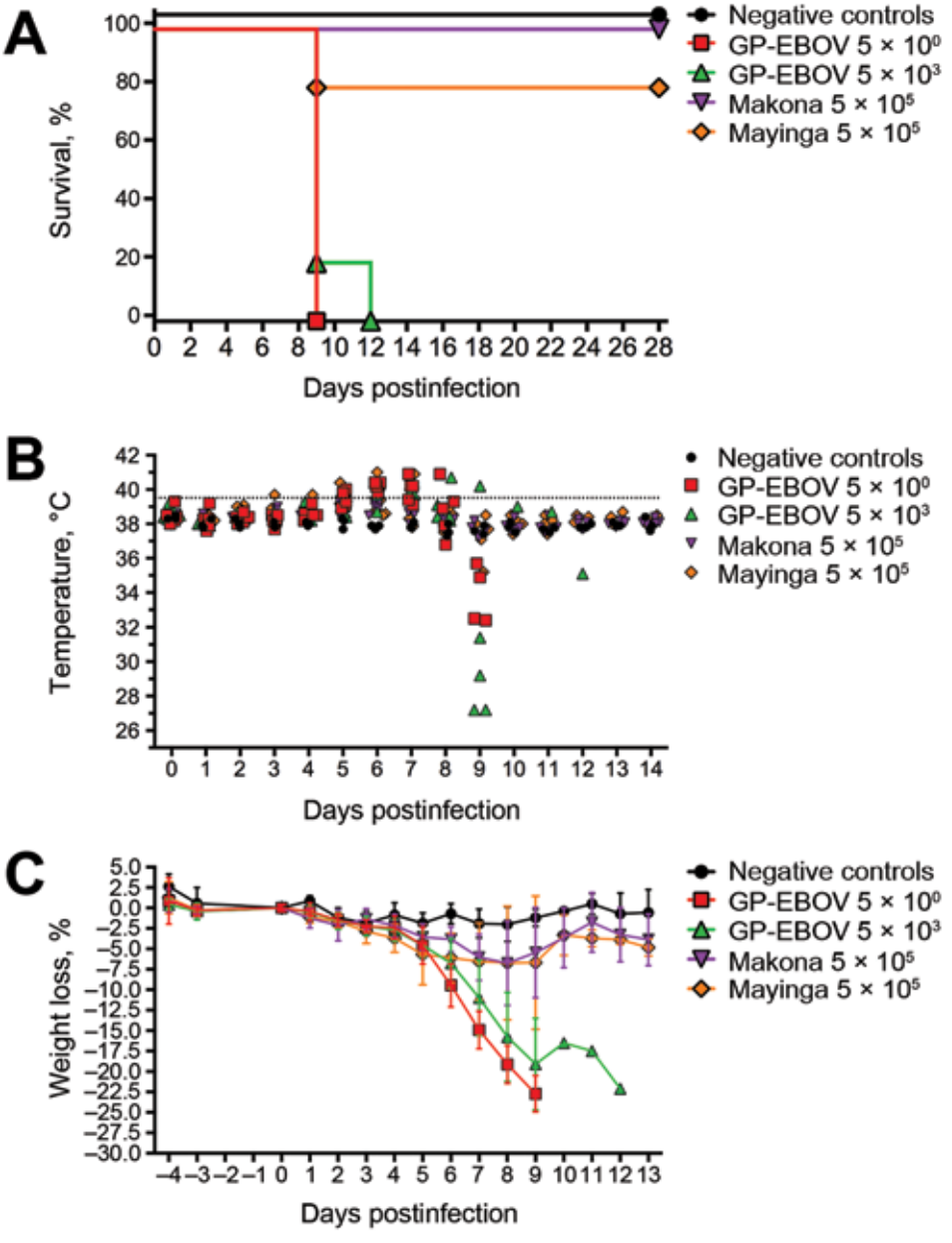


**Figure.** Clinical course of guinea pigs infected with guinea pig–adapted Ebola virus (GP-EBOV), wild-type EBOV Makona, and wild-type EBOV Mayinga, by number of days postinfection. A) Percentage of animals that survived. B) Subcutaneous microchip temperature. Dotted line indicates upper limit of reference temperature range for guinea pigs. C) Weight loss from 0 days postinfection.

**Table.** qRT-PCR results for EBOV nucleoprotein from guinea pig oral swab and blood samples collected, by number of days postinfection*

| ID | Virus type | Dose, $TCID_{50}$/mL | Sample type, blood or oral/blood† | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | D0 | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D8 | D9 | D10 | D11 | D12 | D14 |
| 1 | GP-EBOV | $5.0 \times 10^0$ | – | – | – | –/– | – | – | + | + | ++ | +++/++++ | NS | NS | NS | NS |
| 2 | GP-EBOV | $5.0 \times 10^0$ | – | – | – | –/– | – | – | + | ++ | +++ | +++/+++++ | **++++** | **++++** | **+++** | **++++** |
| 3 | GP-EBOV | $5.0 \times 10^0$ | – | – | – | –/– | – | – | + | +++ | ++++ | ++++/+++++ | **+++++** | **++++** | **+++** | **++++** |
| 4 | GP-EBOV | $5.0 \times 10^0$ | – | – | – | –/– | – | – | ++ | +++ | +++ | ++++/+++++ | **++++** | **++++** | **+++** | **++++** |
| 5 | GP-EBOV | $5.0 \times 10^0$ | – | – | – | –/NS | – | – | – | ++ | +++ | ++++/+++++ | **++++** | **++++** | **++++** | **++++** |
| 6 | GP-EBOV | $5.0 \times 10^3$ | – | – | – | –/– | – | – | + | +++ | +++ | +++/++++ | **+++** | **+++** | **++** | **+++** |
| 7 | GP-EBOV | $5.0 \times 10^3$ | – | – | – | –/+ | – | – | ++ | +++ | +++ | +++/+++++ | **++++** | **++++** | **+++** | **++++** |
| 8 | GP-EBOV | $5.0 \times 10^3$ | – | – | – | –/NS | – | – | ++ | ++ | +++ | +++/++++ | **++++** | **+++++** | **+++** | **++++** |
| 9 | GP-EBOV | $5.0 \times 10^3$ | – | – | – | –/– | – | – | – | – | – | – | + | + | +/+++ | **++** |
| 10 | GP-EBOV | $5.0 \times 10^3$ | – | – | – | –/+++ | – | – | ++ | ++ | +++ | ++++/+++ | **++++** | **+++++** | **+++** | **+++** |
| 11 | WT-Makona | $5.0 \times 10^5$ | – | – | – | –/– | – | – | – | – | – | – | – | – | – | – |
| 12 | WT-Makona | $5.0 \times 10^5$ | – | – | – | –/++ | – | – | – | – | – | – | – | – | – | – |
| 13 | WT-Makona | $5.0 \times 10^5$ | – | – | – | –/– | – | – | – | – | – | – | – | – | – | – |
| 14 | WT-Makona | $5.0 \times 10^5$ | – | – | – | –/++ | – | – | + | + | + | – | – | – | – | – |
| 15 | WT-Makona | $5.0 \times 10^5$ | – | – | – | –/+ | – | – | – | – | – | – | – | – | – | – |
| 16 | WT-Mayinga | $5.0 \times 10^5$ | – | – | – | –/++ | – | – | – | – | – | – | – | – | – | – |
| 17 | WT-Mayinga | $5.0 \times 10^5$ | – | – | – | –/– | – | – | + | – | – | – | – | – | – | – |
| 18 | WT-Mayinga | $5.0 \times 10^5$ | – | – | – | –/NS | – | + | – | + | + | – | – | – | – | – |
| 19 | WT-Mayinga | $5.0 \times 10^5$ | – | – | – | –/– | – | – | – | – | – | – | – | – | – | – |
| 20 | WT-Mayinga | $5.0 \times 10^5$ | – | – | – | –/+++ | – | – | – | ++ | + | +/+ | **++** | **++** | **++** | **+++** |
| 21 | Neg control | DMEM | – | – | – | –/NS | – | – | – | – | – | – | – | – | – | – |
| 22 | Neg control | DMEM | – | – | – | –/– | – | – | – | – | – | – | – | – | – | – |
| 23 | Neg control | DMEM | – | – | – | –/– | – | – | – | – | – | – | – | – | – | – |

*Boldface indicates postmortem samples. Gray shading indicates period of overt clinical disease (days 6–9 postinfection). D, days postinfection; DMEM, Dulbecco's Modified Eagle's medium; EBOV, Ebola virus; GP, guinea pig–adapted; ID, identification number; Neg, negative; NS, not sampled; qRT-PCR, quantitative reverse transcription PCR; $TCID_{50}$, 50% tissue culture infectious dose; WT, wild-type.
†EBOV RNA copies/mL: –, negative; +, $10^0$–$10^1$; ++, $10^2$; +++; $10^3$; ++++, $10^4$; +++++, $10^5$–$10^6$.

WT-EBOV-Mayinga. At 6 dpi, coinciding with the time of overt clinical signs of disease (i.e., fever, weakness, anorexia, and ruffled fur), qRT-PCR of oral swab samples detected EBOV RNA in 8 (73%) of 11 animals in which fatal illness developed and in 10 (50%) of 20 infected animals. EBOV RNA was detected by qRT-PCR in all postmortem swab samples.

## Conclusions

Our data suggest that oral swab samples obtained early in the course of infection, before death, are not a reliable method for diagnosing infection with EBOV. Paired oral swab and blood samples collected at 3 dpi and at time of euthanasia showed that sensitivity of oral swab samples was low compared with the sensitivity of traditional blood samples. Testing of oral swab samples did not indicate infection until 3 days after EBOV RNA was detectable in blood samples, with the exception of 1 animal in which oral swab samples revealed viral RNA 2 days after the blood sample. At the time of overt clinical disease, the utility of oral swab samples for diagnostics improved but was not completely consistent with infection until postmortem time-points. Our studies also enabled us to investigate whether the virulence of the WT-EBOV-Makona variant in guinea pigs was as low as that of the prototypic WT-EBOV-Mayinga variant. As shown in previous studies (*3,5,6*), WT-EBOV is less pathogenic than GP-EBOV, regardless of variant, in this animal model.

Investigating the utility of oral swab samples for diagnosing EVD in humans is challenging because paired blood and oral swab samples are rarely available and because the timing of sample collection relative to onset of disease and course of infection is often estimated. Although EVD in the nonhuman primate model mimics many aspects of the disease in humans, sampling from nonhuman primates in an experimental setting is problematic because of the species' temperament, which requires anesthesia during specimen collection and venipuncture. The guinea pig model of EVD (*3,5,6*) offers the convenience of daily oral swab sampling without the need for anesthesia.

Although suggestive, as with any animal model system, when extrapolating these data to human diagnostics, the effect of potential differences in oral milieus (e.g., saliva composition and oral cavity tissue structure) must be considered. In the future, additional studies that use paired oral swab and blood samples from humans would provide information for continued discussion of antemortem swab sampling as a useful diagnostic modality of EVD in humans.

Our data support the use of oral swab samples as a sensitive modality for postmortem diagnostics; however, the utility of oral swab samples under field conditions, especially those collected before death, may decrease because of inherent problems with sampling techniques and specimen handling conditions (i.e., delays in transport and storage at typically high ambient temperatures). Despite these considerations, oral swab sample collection

could be a useful sampling strategy for humans and animals with unknown causes of death when EVD is suspected and when other types of samples are more prohibitive to obtain.


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# Spatiotemporal Patterns of Schistosomiasis-Related Deaths, Brazil, 2000–2011

**Francisco Rogerlândio Martins-Melo, Marta Cristhiany Cunha Pinheiro, Alberto Novaes Ramos Jr, Carlos Henrique Alencar, Fernando Schemelzer de Moraes Bezerra, Jorg Heukelbach**

We analyzed spatiotemporal patterns of 8,756 schistosomiasis-related deaths in Brazil during 2000–2011 and identified high-risk clusters of deaths, mainly in highly schistosomiasis-endemic areas along the coast of Brazil's Northeast Region. Schistosomiasis remains a neglected public health problem with a high number of deaths in disease-endemic and emerging focal areas.

Schistosomiasis is a neglected tropical disease (NTD) caused by infection with *Schistosoma* spp. trematodes and a public health problem worldwide, mainly in areas without access to safe drinking water and adequate sanitation (*1*,*2*). Brazil is the most heavily affected country in the Americas (*1*), with about 2.5 million–6 million infected persons (*3*) and 700–800 deaths are reported annually (*4*). The disease's continued expansion because of human migration from schistosomiasis-endemic to -nonendemic areas means schistosomiasis is increasingly considered an emerging disease in Brazil (*5*). Using different spatial analytical approaches, we examined spatiotemporal patterns and determined high-risk clusters for schistosomiasis-related deaths in Brazil.

## The Study

We analyzed death certificate data obtained from the Brazilian Mortality Information System (http://tabnet.datasus.gov.br/cgi/sim/dados/cid10_indice.htm) and used the 5,565 municipalities of residence in Brazil as geographic units of analysis. We included deaths occurring during 2000–2011 for which schistosomiasis (code B65, International Classification of Diseases, Tenth Revision [ICD-10]) was recorded as underlying or associated (contributing) causes of death (multiple causes of death) (*6*). Deaths with unknown municipality of residence were excluded. Population data at the municipality level were obtained from the Brazilian Institute of Geography and Statistics (http://tabnet.datasus.gov.br/cgi/deftohtm.exe?ibge/cnv/popuf.def).

To minimize random variations, especially in municipalities with small populations and rare events, we calculated average annual death rates (per 100,000 inhabitants) at the municipality level over the entire period (average annual number of deaths/population size during the middle of the study period). We then calculated smoothed death rates by using the local empirical Bayes method (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/14-1438-Techapp1.pdf). Presence of global and local spatial autocorrelation was evaluated by using Global Moran's I and Local Moran's I statistics (*7*), respectively (online Technical Appendix). A retrospective space-time scan statistic (*8*) was used to identify statistically significant high-risk spatiotemporal clusters (online Technical Appendix). Primary (i.e., most likely) and secondary clusters were detected by using the log-likelihood ratio test; clusters with maximum log-likelihood ratios were considered primary.

A total of 12,491,280 deaths were recorded in Brazil for 2000–2011. Schistosomiasis was identified in 8,756 deaths (0.07%), as an underlying cause in 6,319 (72.2%) and as an associated cause in 2,437 (27.8%) deaths. The nationwide average annual crude rate of death atttibuted to schistosomiasis (for underlying and associated causes) was 0.39 deaths (95% CI 0.37–0.42) per 100,000 inhabitants. Of 5,565 municipalities, ≈1,225 (≈22%) recorded ≥1 schistosomiasis-related death. Spatial distribution of average annual crude and smoothed death rates at the municipal level showed a concentration of municipalities with higher death rates (>1.0 death/100,000 inhabitants) along the east coast of Brazil's Northeast Region, extending to the states of Minas Gerais and Espírito Santo (Figure 1, panels A, B).

Global Moran's I index showed significant positive spatial autocorrelation (0.32, $p<0.01$). Local Moran's I identified high-risk clusters (classified as "High/High") of schistosomiasis-related deaths, corresponding mainly to municipalities with high rates shown in the descriptive maps (Figure 2, panel A). As with the concentration of high death rates, major high-risk clusters included a large geographic area on the east coast of the Northeast region (Figure 2, panel A).

Scan space-time analysis identified 3 spatiotemporal high-risk clusters (Figure 2, panel B; Table). Primary

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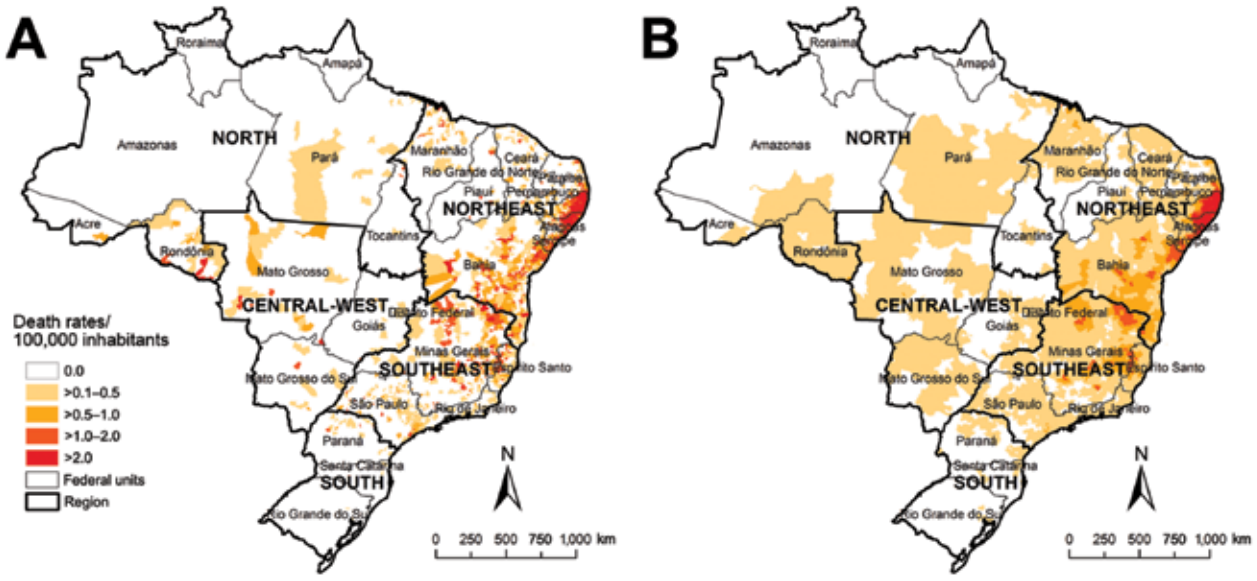


**Figure 1.** Spatial distribution of average annual crude (A) and Bayesian-smoothed (B) rates of schistosomiasis-related deaths, by municipality of residence, Brazil, 2000–2011. Empirical Bayesian smoothing estimates of rates of schistosomiasis-related deaths were performed by using TerraView software version 4.2 (Instituto Nacional de Pesquisas Espaciais, São Paulo, Brazil). Data were mapped by using ArcGIS software version 9.3 (Esri, Redlands, CA, USA). In 2010, Brazil was divided into 5 geographic regions (South, Southeast, Central-West, North, and Northeast), 27 Federal Units (26 states and 1 Federal District), and 5,565 municipalities.

clusters were detected during 2001–2006 and represented 2,150 deaths in 191 municipalities distributed in 3 states in the Northeast region. The relative risk was 12.96 ($p<0.01$), and the annual crude rate was 4.0 deaths/100,000 inhabitants. Secondary clusters were located in the Southeast and Northeast regions (Figure 2, panel B; Table).

## Conclusions

In this nationwide population-based study in Brazil, we found a heterogeneous geographic pattern of schistosomiasis-related deaths. Independently from the spatial statistical approach, high-risk clusters for schistosomiasis-related deaths were identified mainly in the highly

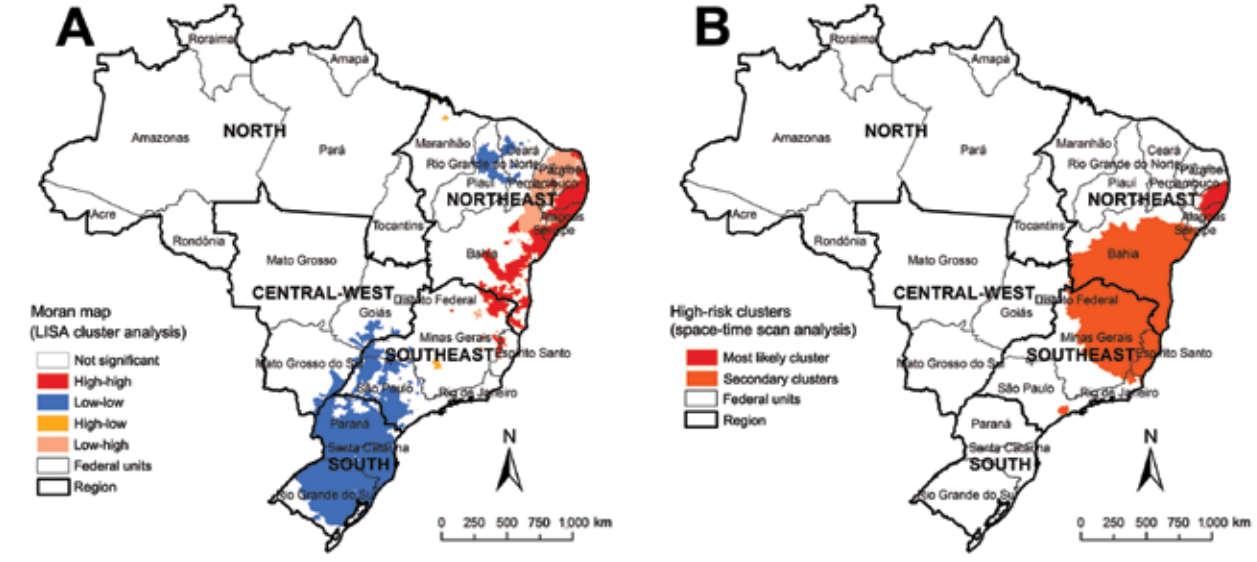


**Figure 2.** Spatial and spatiotemporal cluster analysis of rates of schistosomiasis-related deaths, by municipality of residence, Brazil, 2000–2011. A) LISA cluster analysis (Moran Map), based on Local Moran's I index. B) Scan space-time clusters analysis, calculated by using Kulldorff's scan statistics with SaTScan software version 9.1.1 (Harvard Medical School, Boston, MA, USA; Information Management Service, Silver Spring, MD, USA). Mapping and calculation of autocorrelation spatial analysis were conducted using ArcGIS software version 9.3 (Esri, Redlands, CA, USA). LISA, Local Index of Spatial Association.

**Table.** Significant spatiotemporal clusters of schistosomiasis-related deaths as defined by space-time scan statistic, by municipality of residence, Brazil, 2000–2011*

| Cluster† | Period | No. munis. | States | Region(s) | Radius, km | Death rate‡ | No. observed/no. expected | LLR | RR | p value |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2001–2006 | 191 | Paraiba, Pernambuco, Alagoas | Northeast | 179.3 | 4.0 | 2,150/214.6 | 3,257.52 | 12.96 | <0.001 |
| 2 | 2006–2011 | 996 | Sergipe, Bahia, Goiás, Minas Gerais, Espírito Santo, Rio de Janeiro | Northeast, Central-West, Southeast | 688.8 | 0.6 | 1,161/734.2 | 116.79 | 1.69 | <0.001 |
| 3 | 2000–2005 | 27 | São Paulo | Southeast | 38.7 | 0.5 | 572/427.9 | 23.16 | 1.36 | <0.001 |

*Space-time scan statistic is described in the online technical appendix (http://wwwncd.cdc.gov/EID/article/21/10/14-1438-Techapp1.pdf). LLR, log-likelihood ratio test; munis, municipalities; RR, relative risk for the cluster compared with the rest of the country.
†The most likely or primary clusters (1) and secondary clusters (2 and 3) were detected by the LLR. The most likely cluster was defined as the one with the maximum LLR.
‡Average annual rate of death for schistosomiasis per 100,000 inhabitants during the cluster period.

schistosomiasis-endemic areas along the east coast of the Northeast Region, particularly in the states of Alagoas, Pernambuco, Sergipe, and Bahia and extending north of Minas Gerais and Espírito Santo States in the Southeast (*4,9,10*). These areas have ecologic and geographic conditions favorable to schistosomiasis: presence and proliferation of the intermediate snail host, poor living conditions, and inadequate sanitation (*10*). Reducing severe forms of schistosomiasis will require controlling transmission by implementing measures such as promoting basic sanitation and health education (*4,11*).

We also identified high rates of schistosomiasis-related deaths in areas where the disease is not endemic and has no focal transmission. The continuing emergence of schistosomiasis, characterized by the appearance of new foci in nonendemic areas and by urbanization of the disease, may be related to internal migration, increasing urban agglomeration, wide distribution of intermediate hosts, and discontinuation of disease control measures (*9*). High levels of internal migratory movement, the spread of snail intermediate hosts, and poor sanitary conditions increase the risk for establishing new foci in Brazil (*9,12*). For example, Rondônia state in North Brazil recorded increasing numbers of confirmed cases in recent years (*13*). Most cases and deaths in this state were not autochthonous but were identified in migrants coming from schistosomiasis-endemic regions of Brazil (*13*). The presence of potential intermediate hosts has been confirmed in Rondônia, increasing the possibility that the disease will establish there (*12,13*). In other regions of the world, transmission seems to establish in non–disease-endemic areas; on the island of Corsica (France), several tourists have been infected with *Schistosoma haematobium* while bathing in local rivers (*14*).

Although schistosomiasis is a disease typical of poor rural areas, intensified urbanization in recent decades has led to increasing numbers of urban cases and deaths (*11,15*). Municipalities that recorded the highest number of deaths were concentrated in Brazilian state capitals, especially in São Paulo (São Paulo State), Recife (Pernambuco State), Maceió (Alagoas State), and Belo Horizonte (Minas Gerais State). Most cases probably originated with persons coming from schistosomiasis-endemic rural areas and migrating to capital cities and metropolitan regions in search of improved living conditions and increased access to specialized health services (*11*).

Furthermore, development and management of water resources projects can introduce schistosomiasis into areas not previously endemic for the disease (*2*). The transposition of the largest river in the Northeast Region (São Francisco River), set to begin in 2016, may contribute to disease outbreaks through dispersion of intermediate hosts to areas not previously schistosomiasis endemic and through increased migratory activities of construction workers and their families (*4*).

Our study is subject to limitations. Because we used secondary death data, deaths may be underreported (*4*), despite progress achieved in registration of deaths (estimated proportion of deaths reported increased from 91.0% in 2000 to 94.2% in 2011; http://tabnet.datasus.gov.br/cgi/idb2012/a1801b.htm). Furthermore, schistosomiasis as an underlying cause of death may be underreported because it could be coded as a complication or illness associated with schistosomiasis (e.g., gastrointestinal bleeding, portal hypertension, esophageal varices) (*4,11*). To reduce this error, we collected information from data showing multiple causes of death (underlying and associated causes) and identified all death certificates that mentioned schistosomiasis. In addition, identifying areas of high transmission of disease by using death data must be approached with care. Schistosomiasis is a chronic disease, and death may result from an infection acquired many years earlier (*4*). Because of geographic migration of infected persons, place of residence at time of death may not be the place where the infection was acquired (*5*). Another limitation is the uncertainty of population estimates during intercensus years used in calculations of rates, especially estimates for years far from census years (2000 and 2010).

Our results indicate spatiotemporal heterogeneity of schistosomiasis-related deaths in Brazil over a 12-year period. High-risk clusters were located mainly in highly schistosomiasis-endemic areas. Disease control programs should increase geographic coverage, intensify and focus efforts to reduce transmission and prevent severe illnesses and deaths, and prevent establishment of schistosomiasis in areas where it is not yet endemic.

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# Taeniasis among Refugees Living on Thailand–Myanmar Border, 2012

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We tested refugee camp residents on the Thailand–Myanmar border for *Taenia solium* infection. Taeniasis prevalence was consistent with that for other disease-endemic regions, but seropositivity indicating *T. solium* taeniasis was rare. Seropositivity indicating cysticercosis was 5.5% in humans and 3.2% in pigs. Corralling pigs and providing latrines may control transmission of these tapeworms within this camp.

Infection with the adult form of *Taenia solium* tapeworms, known as taeniasis, results from consuming undercooked or raw pork that is contaminated by this parasite (http://www.cdc.gov/parasites/taeniasis/gen_info/faqs.html). Though these infections are often asymptomatic, infection with tapeworm eggs can progress to a larval infection of the central nervous system, known as neurocysticercosis, that poses a serious public health hazard. Neurocysticercosis is a major cause of acquired epilepsy in the developing world (*1–3*) and an emerging public health issue in the United States because of emigration from and travel to areas in Latin America, Asia, and Africa where the disease is endemic (*4–6*). Multiple cases of neurocysticercosis have been reported among resettled refugees from Myanmar, one of the largest refugee groups recently resettled in the United States (*7–10*). A recent seroprevalence survey showed that 1 in 4 refugees from Myanmar has antibodies against *T. solium* cysts (*8*), which suggests that *T. solium* infection might be endemic to Myanmar or camps in Thailand where refugees reside before they are resettled. However, because little is known about transmission of this zoonotic parasite in that region, opportunities for disease control and prevention have been limited.

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## The Study

In October 2012, we conducted a cross-sectional study in the Ban Mai Nai Soi refugee camp on the Thailand–Myanmar border. This camp was established in 1996 to house persons displaced by conflicts between ethnic minorities and the Myanmar government. Many of these refugees subsequently resettle in the United States. Approximately 13,591 persons in roughly 3,000 households lived in the camp as of October 2012 (*11*). Camp residents live in closely packed bamboo housing; many have small yards with an enclosed pit latrine. Residents do not have access to electricity and obtain treated water from communal water stations located throughout the camp. Domestic pig rearing is common, and corral use is mandated by camp authorities.

We randomly selected participants from all houses in the camp by using hand-drawn maps provided by the camp's governing committee and invited all household residents, regardless of age, to participate. We interviewed all consenting residents using a standard questionnaire in Karenni, their primary language, and collected a fecal sample and blood sample from them. We attempted to interview all participants directly, but allowed parents to answer questions for their young children. We examined whole fecal samples macroscopically in a field laboratory for *Taenia* sp. proglottids or scoleces (i.e., segments or anterior ends) but did not use morphologic characteristics to identify the species of the recovered tapeworm material. Fecal and blood samples were transported on ice each day to the laboratory at Chiang Mai University for further processing. Fecal samples were concentrated by sedimentation and examined by light microscopy for *Taenia* sp. eggs. Pig blood was analyzed by enzyme-linked immunoelectrotransfer blot (EITB) for antibodies against *T. solium* cysticerci (*12*). Human blood was analyzed by EITB for antibodies against recombinant antigens specific to *T. solium* tapeworms (rES33) and cysticerci (rT24) (*12,13*). The sensitivity and specificity of these tests are 97% and 100%, respectively, for rES33, and 94% and 98%, respectively, for rT24. Participants with taeniasis were given a single oral dose of niclosamide with bisacodyl to assist

in tapeworm elimination (additional methods are provided in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/14-1657-Techapp1.pdf).

We interviewed 738 persons in 205 randomly sampled households, representing roughly 5% (738/13,591) of the total camp population (Table). Our sample included a higher proportion of girls and women (394, 53.4%), and participants' mean age was 23.7 years (SD ± 19.8), ranging from a few months to 84 years of age. Participants had a mean of 2.8 years of education (SD ± 3.6) and had lived in the camp for an average of 9.9 years (SD ± 5.9). Most participants were unemployed (575, 80.0%) and owned at least 1 pig (549, 74.4%); none owned a cow. Among the 138 (67%) households in which pigs were raised, all but 1 (≈100%) household maintained their pigs in a corral. Approximately one quarter of participants reported eating raw pork (190, 26.1%) and raw beef (178, 24.6%).

Fecal samples were collected from 552 (75%) participants. Of these, 18 (3.3%) participants tested positive for taeniasis (i.e., eggs or proglottids were detected by microscopy), ranging from 1 (0.6%) person <10 years of age to 7 (11.7%) persons >54 years of age (Figure). After accounting for intrahousehold clustering and sampling weight, the prevalence of taeniasis as determined by microscopy was 2.9% (95% CI 1.4%–4.3%). Among the 671 persons with blood sample results, only 1 (0.1%) had serologic test results indicating *T. solium* taeniasis. This person was negative for *Taenia* sp. eggs or proglottids via fecal microscopy and was not seropositive for antibodies indicating *T. solium* cysticercosis.

Blood samples were collected from 671 (91%) participants. Of these, 29 (4.3%) were seropositive for antibodies against *T. solium* cysticerci by using EITB rT24, ranging from 6 (2.9%) persons <10 years old to 10 (15%) persons >54 years old (Figure). After accounting for household clustering and sampling weight, seroprevalence was 5.5% (95% CI 2.9%–8.1%). Of the 258 pigs whose blood we collected, 11 (4.3%) were seropositive for antibodies against *T. solium* cysticerci by EITB lentil-lectin purified glycoprotein. After adjustment for household clustering and sampling weight, 3.2% (95% CI 0.8%–5.5%) were seropositive.

**Table.** Characteristics of refugees living on the Thailand–Myanmar border who participated in taeniasis study and their households, 2012*

| Characteristic | Finding |
|---|---|
| Refugees, n = 738 | |
| Age, y, median (IQR); mean (SD) | 18 (7–35); 23.7 (19.8) |
| Education, y, median (IQR); mean (SD) | 1 (0–5); 2.8 (3.6) |
| Lived in camp, y, median (IQR); mean (SD) | 11 (4–16); 9.9 (5.9) |
| Female sex, no. (%) | 394 (53.4) |
| Unemployed, no. (%) | 575 (80.0) |
| Households, n = 205 | |
| No. residents, median (IQR); mean (SD) | 4 (4–6); 4.3 (1.9) |
| Main floor elevated, no. (%) | 169 (83.7) |
| Main floor cement or bamboo, no. (%) | 198 (98.5) |
| Latrine in yard, no. (%) | 170 (83.7) |
| Water runs from tap to house, no. (%) | 35 (17.2) |
| At least 1 pig, no. (%) | 138 (67.3) |

*IQR, interquartile range.

## Conclusions

Taeniasis is relatively common among residents of the Ban Mai Nai Soi refugee camp. The overall prevalence of 2.9% is consistent with estimates from other regions where *Taenia* sp. are endemic. Although we did not definitively identify the species of taeniasis present, serologic results suggest that *T. solium* is not the dominant *Taenia* species in this population. We found only 1 person with serum antibodies indicating *T. solium* taeniasis using an assay that is 100% specific for *T. solium*. The prevalence of antibodies indicating *T. solium* cysticercosis in humans was also low. Given this combination of findings, *T. asiatica* or *T. saginata* are likely the dominant species in this camp. The risk of acquiring neurocysticercosis in Ban Mai Nai Soi is therefore likely to be relatively low. However, the distribution of *Taenia* sp. tapeworms varies considerably among geographic areas and ethnic groups, so these results might not be generalizable to other camps or communities along the Thailand–Myanmar border.

The universal availability of sanitary infrastructure and wholesale adoption of animal corralling within the camp limit the conditions necessary for the *T. solium* tapeworm to complete its lifecycle. These prevention measures might control transmission despite the pervasive poverty and common practice of eating raw pork. Sanitation and pig restraint within the camps and surrounding communities might reduce the risk for *T. solium* taeniasis and neurocysticercosis in this region.

This study has limitations. Because we collected only 1 fecal sample from each participant, we might have underestimated the prevalence of taeniasis. Although we recovered

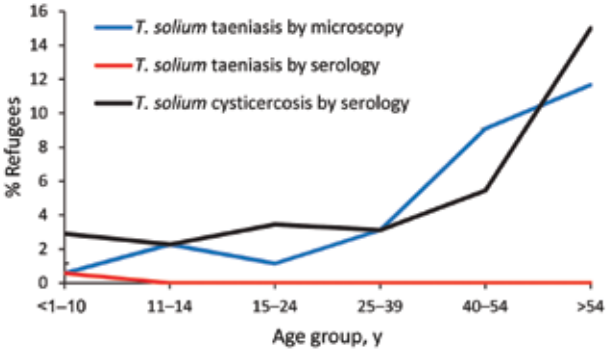


**Figure.** *Taenia solium* taeniasis and cysticercosis in refugees living on the Thailand–Myanmar border, 2012. Taeniasis by microscopy indicates presence of *T. solium* eggs or proglottids in stool.

tapeworm material from fecal samples, we were not able to perform any molecular analyses, so the exact species of *Taenia* remains unconfirmed. Many of the variables collected were self-reported and therefore subject to participants' recall bias.

Additional epidemiologic studies are needed to improve understanding of the distribution of *Taenia* sp. infections in this region and the effects of associated neurologic disease. Screening and treatment for taeniasis among refugees before resettlement might also reduce the risk of further transmission in the receiving country.

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# Public Health Response to *Aedes aegypti* and *Ae. albopictus* Mosquitoes Invading California, USA

**Charsey Cole Porse, Vicki Kramer, Melissa Hardstone Yoshimizu, Marco Metzger, Renjie Hu, Kerry Padgett, Duc J. Vugia**

*Aedes aegypti* and *Ae. albopictus* mosquitoes, primary vectors of dengue and chikungunya viruses, were recently detected in California, USA. The threat of potential local transmission of these viruses increases as more infected travelers arrive from affected areas. Public health response has included enhanced human and mosquito surveillance, education, and intensive mosquito control.

Two mosquito species, Aedes aegypti and Ae. albopictus, are principal vectors for dengue virus (DENV) and chikungunya virus (CHIKV), both infectious to humans. These mosquitoes are daytime biters, need only small water containers to propagate, and are difficult to eradicate.

Dengue, caused by DENV infection, is a viral febrile illness characterized by headache, retro-orbital pain, myalgia, arthralgia, rash, and potentially hemorrhagic manifestations. Most persons with dengue are asymptomatic, but the virus can still be transmitted to mosquitoes that bite them (*1*). Reported dengue-associated illnesses and deaths are high, and increasing worldwide; an estimated 50–100 million cases occur annually (*2*). Since 2000, DENV transmission has occurred in areas of the United States where *Ae. aegypti* and *Ae. albopictus* are established, and outbreaks have occurred in Florida, Texas, and Hawaii (*3*).

Chikungunya fever, caused by CHIKV infection, is also a viral febrile illness and is characterized by severe polyarthralgia (*4*,*5*). Most persons infected with CHIKV are symptomatic (*4*,*6*). An ongoing outbreak of CHIKV began in late 2013 in the Caribbean and has spread to 43 countries or territories throughout Central America, South America, North America, and the Caribbean (*7*). As of 2014, more than 2,000 cases of chikungunya fever had been reported in the United States; except for 11 locally acquired cases in Florida, all cases were acquired abroad (*8*).

DENV- and CHIKV-infected returned travelers or visitors presumably imported the viruses and served as sources for local US outbreaks (*3*,*6*). The established presence of *Ae. aegypti* and *Ae. albopictus* mosquitoes in the southeastern United States poses a threat for potential transmission of DENV and CHIKV to local residents as infected, possibly viremic, travelers return or arrive from countries where transmission is ongoing. This threat is now expanding to California. In several regions of the state, imported cases of DENV and CHIKV infections have been documented where recent detection and persistence of *Ae. albopictus* and *Ae. aegypti* mosquitoes concurrently occur.

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## The Study

In California, surveillance and control of mosquitoes and mosquitoborne diseases is a collaborative effort involving vector-control agencies, local health departments, the California Department of Public Health (CDPH), and the University of California, Davis, Center for Vectorborne Diseases (CVEC). Vector-control agencies perform mosquito surveillance and control by using specialized traps and products targeting adult and immature mosquitoes. Local health departments conduct follow-up of reported human cases. CDPH provides confirmation of mosquitoborne disease cases in humans, mosquito identification, and consultation and assistance in responding to invasive mosquitoes and disease trends. CVEC tests *Ae. aegypti* and *Ae. albopictus* mosquitoes for DENV, CHIKV, and West Nile virus.

Before 2011, *Ae. aegypti* and *Ae. albopictus* mosquitoes were rarely detected in California and were not known to persist. Of these previous detections, the largest was an infestation of *Ae. albopictus* mosquitoes that were imported with "lucky bamboo" from China into Los Angeles County in 2001 (*9*). In 2011, *Ae. albopictus* mosquitoes were detected in El Monte, Los Angeles County, California, and genetic analysis indicated they may have been remnants of the 2001 importation (*10*). Since 2011, enhanced mosquito surveillance has documented the persistence and spread of *Ae. albopictus* mosquitoes to 12 surrounding cities within Los Angeles County.

In 2013, *Ae. aegypti* mosquitoes were detected in Fresno, Madera, and San Mateo Counties, California; analysis indicated they were genetically most similar to *Ae. aegypti* mosquitoes from the southeastern United States (*11*). In 2014, *Ae. aegypti* mosquitoes persisted in those 3 counties and were also detected in Kern, Tulare, Los Angeles, and San Diego Counties. In 2015, *Ae. aegypti* mosquitoes were detected in Imperial, Orange, and Alameda Counties (Figure). Of 1,729 *Ae. aegypti* mosquitoes captured

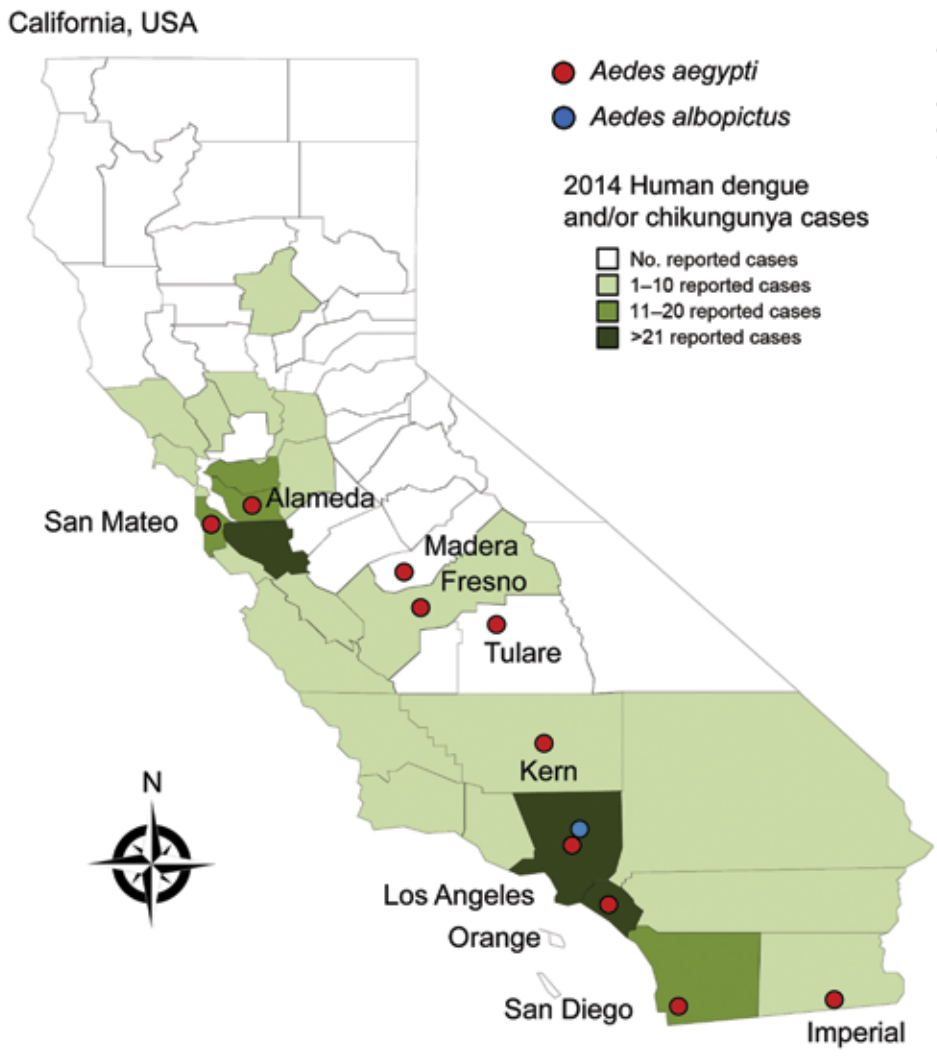


**Figure.** Invasive *Aedes* mosquitoes detected during 2011–2015 and number of imported human cases of dengue, chikungunya fever, or both reported during 2014 in counties in California, USA.

during 2013–2014 and tested at CVEC, none were positive for DENV or CHIKV.

Dengue, but not chikungunya fever, is reportable in California. Nonetheless, CDPH is notified of confirmed and probable chikungunya fever cases. During 2009–2013, CDPH was notified of 3 confirmed chikungunya fever cases, and the reported annual number of dengue cases increased from 36 to 125, all among returned travelers or visitors. In 2014, a total of 141 chikungunya fever and 133 dengue cases were reported, all in persons with recent travel to DENV- or CHIKV-endemic or outbreak areas. Of the 133 dengue case-patients, 98 (74%) were likely viremic while in California, 71 (54%) had illness onset after arrival, and 27 (20%) had illness onset within 5 days before arrival. Of the 141 chikungunya fever case-patients, 93 (66%) were likely viremic in California, 58 (41%) had illness onset after arrival, and 35 (25%) had illness onset within 7 days before arrival. Of these likely viremic patients, 44% (43/98) with dengue and 58% (54/93) with chikungunya fever arrived or returned to a county with an infestation of invasive *Aedes* mosquitoes and, thus, represented a potential risk for virus transmission if bitten by an *Ae. aegypti* or *Ae. albopictus* mosquito.

## Discussion

We document the invasion and persistence in California of *Ae. albopictus* mosquitoes since 2011 and *Ae. aegy*pti mosquitoes since 2013. The risk for local transmission of DENV and CHIKV is currently low because of small numbers of infected travelers arriving in California and limited distribution of invasive *Aedes* mosquitoes in the state. However, the threat for local transmission of these viruses is increasing as more infected, potentially viremic travelers arrive from affected areas. The presence of *Ae. albopictus* and *Ae. aegypti* mosquitoes in southern California is of particular concern because of the state's large population, high number of travelers, and proximity to Mexico. Of the DENV and CHIKV infections reported

in 2014, a total of 59% (162/274) were in southern California residents. In 2014, Mexico reported 32,100 dengue and 155 chikungunya fever cases (*12*,*13*), and the presence of *Ae. aegypti* mosquitoes has been established in Mexican cities along the California–Mexico border (e.g., Mexicali, Tecate, Tijuana) (*14*). The large number of persons crossing the US–Mexico border each day creates another potential source of imported cases of dengue and chikungunya fever.

In response to the threat of increased transmission of these viruses, CDPH has developed and distributed guidance to local public health and vector-control agencies to enhance human case and mosquito surveillance, increase knowledge and awareness of these diseases among the general public and the local medical community, and apply intensive *Aedes* mosquito control and prevention measures (*15*). Continuous enhanced surveillance for human cases of DENV and CHIKV infection and ongoing monitoring of invasive *Aedes* mosquito populations are necessary to gauge the level of risk in various regions of the state. Surveillance and monitoring is also necessary to increase the public health response if an *Aedes* mosquito tests positive for DENV or CHIKV or if a patient without travel consistent for DENV or CHIKV exposure has laboratory-confirmed dengue or chikungunya fever. Improved public awareness will increase reporting of mosquitoes that bite during the day, and improved awareness among health care providers may shorten the time to diagnosis of dengue and chikungunya fever cases. Targeted mosquito surveillance and control are essential for limiting the density and geographic spread of these mosquitos to minimize the risk of a viremic person coming in contact with them. Close collaboration between CDPH and local health departments and vector-control agencies is necessary to obtain accurate and timely travel histories from case-patients and to implement follow-up mosquito surveillance in areas surrounding their residences. Through these public health activities, we hope to keep the risk for local transmission of DENV and CHIKV low while the threat is increasing.

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Dr. Porse is an epidemiologist in the Vector-Borne Disease Section, Infectious Diseases Branch, Division of Communicable Disease Control, California Department of Public Health. Her research interests include epidemiology, mosquitoborne diseases, and public health.

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# Heartland Virus Neutralizing Antibodies in Vertebrate Wildlife, United States, 2009–2014

**Kasen K. Riemersma[1] and Nicholas Komar**

Since its discovery in 2009, the tickborne Heartland virus (HRTV) has caused human illness in Missouri, Oklahoma, and Tennessee USA. To better assess the geographic distribution of HRTV, we used wildlife serology as an indicator. This retrospective evaluation determined that HRTV is widespread within the central and eastern United States.

Heartland virus (HRTV; family *Bunyaviridae*, genus *Phlebovirus*) is an emerging public health threat in the United States. HRTV disease, characterized by severe fever, leukopenia, and thrombocytopenia, was first reported in 2 farmers in northwestern Missouri in 2009 (*1*). Seven additional HRTV disease cases (2 fatal) have been reported in Missouri, Tennessee, and Oklahoma (*2*,*3*). A study of ticks and mosquitoes in northwestern Missouri detected HRTV infections only in *Amblyomma americanum* (lone star tick) and thus implicated this tick as a vector (*4*). The virus was isolated solely from deplete host-seeking nymphs, which presumably were infected as larvae after feeding on a viremic vertebrate host. Because HRTV has yet to be isolated from any wild or domestic animals, the question of vertebrate reservoir(s) remains unanswered. However, high prevalence of seropositive white-tailed deer (*Odocoileus virginianus*) and raccoon (*Procyon lotor*) from northwestern Missouri indicate these species as targets for wildlife serosurveillance (*5*).

The HRTV disease case reports in Tennessee and Oklahoma after the initial case reports in Missouri create the perception that HRTV transmission activity might be dispersing from an origin in northwestern Missouri. However, the geographic range of HRTV activity is unknown. HRTV distribution may mirror the range of the lone star tick, which is distributed throughout most of the central and eastern United States and recently has expanded northward (*6*). To investigate the hypothesis that HRTV activity occurs throughout the range of its putative tick vector, we conducted a retrospective serosurvey of mainly white-tailed deer and raccoon from 19 states within the heart and periphery of the lone star tick range to look for evidence of HRTV activity.

## The Study

Banked blood samples collected from white-tailed deer, raccoon, and (occasionally) moose (*Alces alces*) and coyote (*Canis latrans*) during 2009–2014 were analyzed by plaque-reduction neutralization test for HRTV neutralizing antibodies by using African green monkey kidney (Vero) cell culture. Only samples from healthy live-trapped animals or deceased animals from anthropogenic causes (i.e., hunting, culls, and automobile strikes) were tested. Inclusion of 19 states was opportunistic based on sample availability. We used the plaque-reduction neutralization test to evaluate HRTV seropositivity for white-tailed deer (n = 396), raccoon (n = 949), coyote (n = 61), and moose (n = 22) (Table 1). Samples consisted of whole blood dried onto Nobuto strips (Advantec MFS, Inc., Dublin, CA, USA), bloody body cavity fluids, or hemolyzed whole blood. Nobuto strip samples were eluted to 1:10 serum dilution in phosphate buffer solution in accordance with the manufacturer's instructions. All samples were heat-inactivated at 56°C for 45 min.

We screened the inactivated samples at 1:20 dilution by mixing serum diluted 1:10 with equal volume of titrated HRTV to approximate a challenge dose of 50 PFUs. Treated Vero cells were incubated for 1 h at 37°C, 5% $CO_2$, before applying a nutrient-rich 0.5% agarose overlay. A second overlay containing Neutral Red was applied after 5–7 d of incubation. Viral plaques were counted 6–12 d after inoculation. A neutralization threshold of 70% relative to HRTV-only controls was used to determine positive samples. All screen-positive samples were repeat-tested to confirm results. Samples were considered seropositive if they were confirmed as positive at a dilution of ≥1:40. Comparative neutralization tests with related viruses were not performed, because we had previously found that murine antiserum developed against the other known phleboviruses in the United States—Sunday Canyon virus (*7*), Rio Grande virus (*8*), and Lone Star virus (*9*)—had no appreciable neutralizing activity against HRTV (Table 2). Human antiserum developed against HRTV exhibited weak 1-way neutralization of Lone Star virus and Sunday Canyon virus (Table 2).

Of 1,428 animals, 103 were seropositive: 55 deer, 33 raccoon, 11 coyotes, and 4 moose. Thirteen states had seropositive animals: Florida, Georgia, Illinois, Indiana, Kansas, Kentucky, Maine, Missouri, New Hampshire, North Carolina, Tennessee, Texas, and Vermont (Table 1; Figure 1). Within the 13 states, 20 geographic clusters of seropositive animals were mapped by plotting positive animals by the county where they were collected (Figure 2).

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**Table 1.** Animals screened and confirmed seropositive for Heartland virus neutralizing antibodies, central and eastern United States, 2009–2014

| State | No. (%) counties sampled | Species | No. screened | Confirmed seropositive, no. (%; 95% CI) |
|---|---|---|---|---|
| Alabama | 5 (7) | Raccoon (*Procyon lotor*) | 99 | 0 (0; 0–4) |
| Florida | 34 (51) | White-tailed deer (*Odocoileus virginianus*) | 65 | 4 (6; 2–15) |
| | | Raccoon | 40 | 0 (0; 0–9) |
| Georgia | 1 (1) | White-tailed deer | 104 | 15 (14; 8–23) |
| Illinois | 8 (8) | Coyote (*Canis latrans*) | 25 | 1 (4; 1–20) |
| | | Raccoon | 68 | 0 (0; 0–5) |
| Indiana | 13 (14) | Raccoon | 64 | 2(3; 1–11) |
| Iowa | 6 (6) | Coyote | 2 | 0 (0; 0–5) |
| | | Raccoon | 98 | 0 (0; 0–13) |
| Kansas | 10 (10) | Coyote | 22 | 10 (46; 27–65) |
| Kentucky | 7 (6) | Raccoon | 44 | 4 (9; 4–21) |
| Maine | 6 (38) | White-tailed deer | 63 | 7 (11; 6–21) |
| Missouri | 10 (9) | Coyote | 12 | 0 (0; 0–24) |
| | | White-tailed deer | 2 | 0 (0; 0–66) |
| | | Raccoon | 75 | 10 (13; 7–23) |
| New Hampshire | 7 (70) | Moose (*Alces alces*) | 22 | 4 (18; 5–40) |
| | | White-tailed deer | 58 | 9 (16; 7–27) |
| North Carolina | 2 (2) | White-tailed deer | 32 | 13 (41; 24–59) |
| Ohio | 7 (8) | Raccoon | 94 | 0 (0; 0–4) |
| Pennsylvania | 15 (22) | Raccoon | 81 | 0 (0; 0–5) |
| Tennessee | 7 (7) | Raccoon | 92 | 13 (14; 8–23) |
| Texas | 22 (9) | Raccoon | 85 | 4 (5; 2–12) |
| Vermont | 5 (36) | White-tailed deer | 72 | 7 (10; 5–19) |
| Virginia | 2 (2) | Raccoon | 37 | 0 (0; 0–9) |
| West Virginia | 19 (35) | Raccoon | 72 | 0 (0; 0–5) |
| Total | | | 1,428 | 103 (7; 6–9) |

## Conclusions

We provide evidence of widespread HRTV transmission activity across the central and eastern United States. Of 13 affected states, only Missouri and Tennessee had previous evidence of HRTV activity. A more stringent neutralization threshold of 80% would reclassify 14 positive samples to "equivocal," but the number of positive states would remain unchanged. These findings should encourage clinicians and public health officials to consider HRTV as a potential source of illness throughout the eastern United States.

Surprisingly, seropositive white-tailed deer were detected in northern New England, where established populations of lone star ticks are unknown (*6*). Possible explanations include unreported lone star tick populations, immigration of seropositive deer, alternative tick vectors for HRTV, or presence of a serologically cross-reactive virus. Movement of deer across state boundaries is an unlikely explanation. Extensive lone star tick populations are not reported in neighboring states (*6*), and migration of deer from lone star tick–infested regions is unlikely (*10*). Savage et al. did not detect HRTV RNA in *Dermacentor variabilis*, the American dog tick (*4*), but additional tick species inhabit northern New England. Several tick species are reported to transmit severe fever with thrombocytopenia syndrome virus, a closely related phlebovirus found in eastern Asia (*11*). Further investigation of tick populations and their vector competence for HRTV is warranted, and production of HRTV neutralizing antibodies in response to a serologically similar virus should be investigated. Two new phleboviruses recently detected in *Ixodes* ticks in the northeastern United States are genetically unrelated to HRTV but raise the possibility that additional undiscovered phleboviruses exist (*12*). Severe fever with thrombocytopenia syndrome virus–reactive antibodies in wildlife were reported in Minnesota, also peripheral to the lone star tick geographic range, indicating the likely presence of HRTV or a similar virus there (*13*).

The finding of seropositive moose and coyotes indicates that these mammals are exposed to HRTV in certain situations and might be useful targets for serosurveillance, in addition to deer and raccoon. The full vertebrate host range and the reservoir competence of these mammals for HRTV remains unknown.

The chronology of dispersal of HRTV is unclear. Suggesting that HRTV emerged in northwestern Missouri and

**Table 2.** Lack of detectable cross-neutralization of HRTV by mouse hyperimmune ascites fluids containing high-titered antibodies to LSV, SCV, and RGV*

| Virus (challenge dose, PFU) | $PRNT_{70}$ antibody titers | | | |
|---|---|---|---|---|
| | HRTV | LSV | SCV | RGV |
| HRTV (54) | 160 | <20 | <20 | <20 |
| LSV (214) | 20 | ≥640 | <20 | <20 |
| SCV (220) | 20 | <20 | 320 | <20 |
| RGV (14) | <20 | <20 | <20 | 320 |

*HRTV, Heartland virus; LSV, lone star virus; $PRNT_{70}$, 70% plaque-reduction neutralization test; RGV, Rio Grande virus; SCV, Sunday Canyon virus.

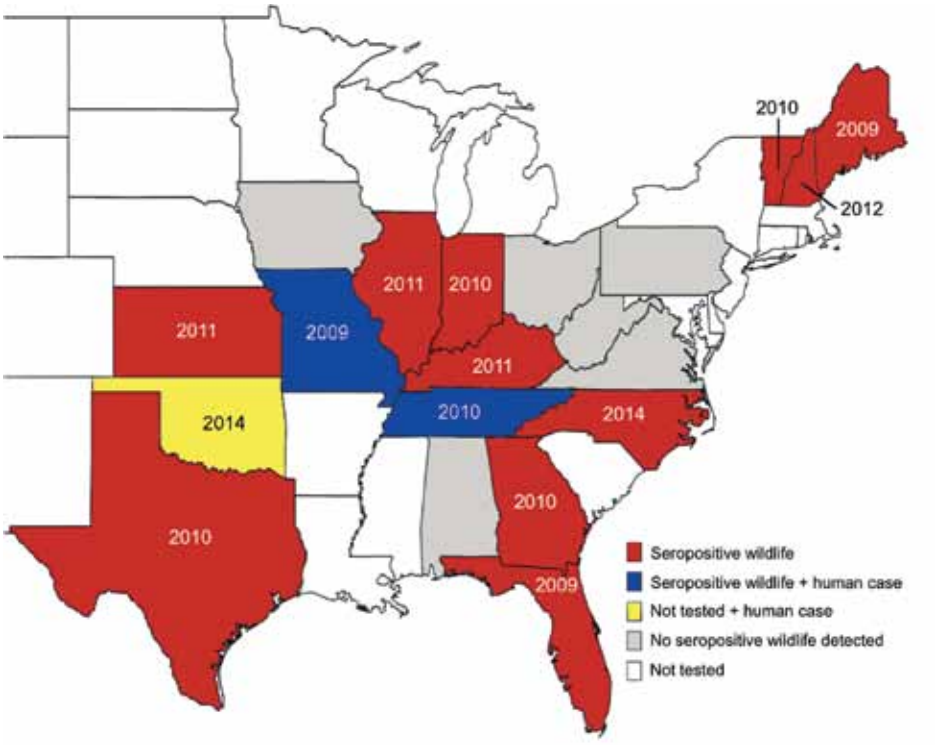


**Figure 1.** State-level distribution of Heartland virus case reports in humans and seropositive wildlife, central and eastern United States, 2009–2014. Year labels indicate the earliest year of detected HRTV activity. Earliest detection was determined by human case reports in Missouri (1 case) and Oklahoma (3 cases) and wildlife serologic data in all other states.

spread to neighboring states to the east and south is overly simplistic. Because animals were sampled at different points of time and space during this study, our data lack robustness to enable comparison of populations over time or between geographic locations. Thus, we are unable to evaluate the dynamics of HRTV spread. Furthermore, the proportions of HRTV-seropositive animal populations lack quantitative value because of our retrospective convenience sampling. Our results simply indicate that HRTV or a very similar virus has circulated in the sampled regions in the recent past and that this activity began as early as 2009. Adult seropositive white-tailed deer were detected in Maine and Florida in

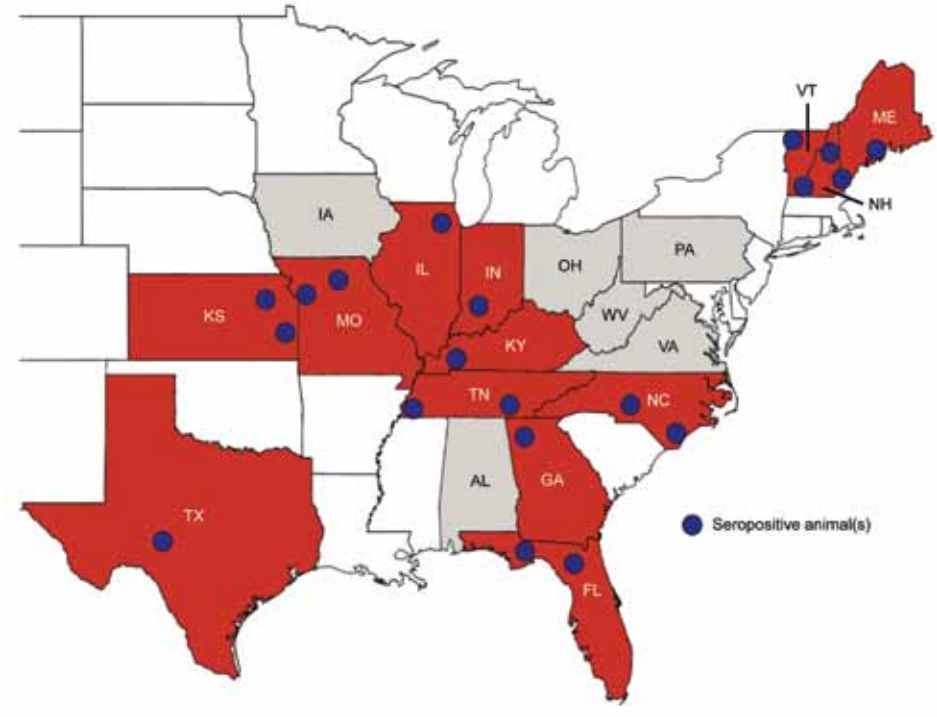


**Figure 2.** Geographic groupings of confirmed seropositive animals for Heartland virus neutralizing antibodies, central and eastern United States, 2009–2014. Twenty groups were identified in 13 states. The geographic locations of the groups were subjectively approximated by the counties where seropositive animals were collected (blue circles). Red indicates states with seropositive animals; gray indicates states in which no seropositive animals were detected. Because of the sampling design, the data are qualitative.

2009, and based on the estimated ages of affected deer (data not shown), the infections could have occurred as early as 2003. A much larger retrospective serosurvey is necessary to elucidate HRTV's history of emergence.


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Dr. Riemersma was a veterinary student in the CDC Epidemiology Elective program at the Division of Vector-Borne Diseases during this study and is currently a PhD student at the University of California, Davis. His research interests include the genetics and ecology of pathogen emergence, with particular interest in vector-borne viruses.

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# Influenza Virus Surveillance in Coordinated Swine Production Systems, United States

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To clarify the epidemiology of influenza A viruses in coordinated swine production systems to which no animals from outside the system are introduced, we conducted virologic surveillance during September 2012–September 2013. Animal age, geographic location, and farm type were found to affect the prevalence of these viruses.

Influenza A viruses (IAVs) are the etiologic agents of acute respiratory disease in many mammalian species. Although originating in wild aquatic birds, IAVs have been successful in crossing the species barrier, and specific subtypes have become endemic among humans and domestic swine populations (*1*). In the United States, influenza was first described in swine herds during the 1918 pandemic and has circulated among domestic pigs for nearly a century (*2*). The ability of swine IAVs to infect humans and cause pandemics such as that of the influenza A(H1N1) virus observed during 2009 (*3*,*4*) and the sporadic transmission of various swine influenza viruses, including H1N1 (*5*), H3N2 (*6*), and variant H3N2 (*7*), are public health concerns and highlight the need for increased vigilance and understanding of IAV epidemiology among swine.

Here we report the results from 13-months of active surveillance of IAV in coordinated swine production systems in the United States. The objectives of this study were 1) to determine the prevalence of IAV within farms in a closed production system and 2) to determine which sampled population is most affected by IAV.

## The Study

Multisite coordinated production systems are the common method of swine production in the United States. These systems consist of multiple farms operating in tandem, with each farm responsible for 1 stage of the production process (Figure 1). These systems are closed, meaning there are no introductions of animals from outside the system. Each farm in the system has a specific purpose: 1) to breed, gestate, farrow, and raise to the point of weaning replacement breeding stock of a specified genotype (multiplier farms [MF]); 2) to raise replacement female pigs, commonly called gilts, to 5–6 months of age for breeding (gilt development farms or units [GDU]); and 3) to breed, gestate, deliver, and raise to the point of weaning piglets specifically for meat production (breed-to-wean farms [BTW]). Gilts from GDUs are moved to MF or BTW farms for breeding. All farms house multiple age cohorts, although in different rooms or buildings. During suckling of piglets on MF and BTW farms, as well as in GDU farms, 1 cohort of piglets differing in age by ≤1 week are housed in a single room/building, and 1 cohort is removed before the entry of the next.

Four coordinated, multisite production systems, each consisting of 1 MF farm that includes both gilts and pigs to produce replacement female stock, 1 GDU farm to raise replacement female gilts from 3 to ≈26 weeks of age, and 4 BTW farms to raise pigs for meat, were selected to monitor the dynamics of IAV transmission in swine breeding herds. Systems are located across the United States. System 1's MF and GDU sites are located in Illinois and its BTW sites in Georgia. Systems 2, 3, and 4 are located entirely in Illinois, Oklahoma, and Nebraska, respectively.

Nasal swab samples were collected from pigs monthly during September 2012–September 2013 from each farm in all 4 systems. The animal type and time of sampling differed on the different farm types. On the MF and BTW farms, samples were collected from 30 piglets at ≈3 weeks of age and from 30 gilts. On the MFs, 30 gilts were sampled before entry to the farm. On the GDU farms, 30 animals at ≈26 weeks of age were sampled before movement to a BTW. In the BTWs, gilts were sampled before breeding (4–8 weeks after arrival) and piglets were sampled immediately before weaning (Figure 1). This strategy enabled assessment of IAV status before the movement of animals to the next stage of production. Laboratory methods are summarized in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/10/14-0633-Techapp1.pdf).

During the 13-month period spanning September 2012–September 2013, a total of 14,954 swab samples were collected and tested for the presence of the IAV matrix gene by real-time reverse transcription PCR. Of the samples collected, 741 (5.0%) tested positive, which is consistent with previous surveillance studies (*8*).

Bivariate analysis found statistically significant correlations between infection and location in Illinois, GDU

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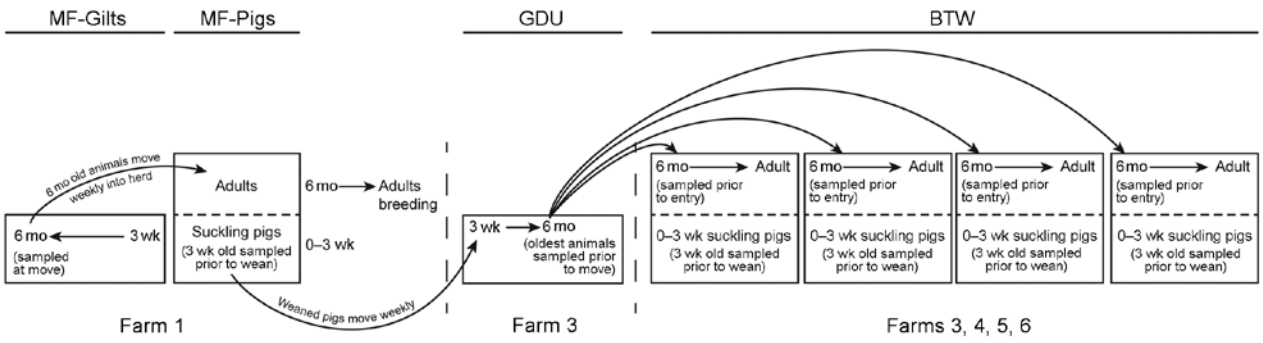


**Figure 1.** Flow of animals through a closed swine production system. Coordinated swine production systems maximize desired animal traits and weight gain. High-quality breeding sows grown and bred in multiplier farms (MF-Gilts) were sampled transfer to commercial gilt development farms (GDU), where they were sampled again at exit (6 months). At ≈3 weeks of age, piglets were sampled before weaning (MF-Pigs). Mature gilts were transported from the GDU to 1 of 4 commercial breed-to-wean (BTW) farms, where samples were collected from gilts before entry and piglets before weaning.

farm type, and system 2 ($p<0.001$ for all), but not for age (Table 1). We then constructed a logistic regression model that assessed the effect of age, system, location, and farm type on having a positive influenza result. Age was statistically significant by this model ($p = 0.004$); the odds ratio for piglets at weaning whose samples tested positive for IAV was 1.3 (95% CI 1.1–1.6) compared to that for gilts (Table 2), which is consistent with previous studies (*9*). However, this finding could be related to interaction between age and state/system. System 2 again had higher odds of positive results (OR 1.7, 95% CI 1.3–2.3) compared with system 1. MF pigs were found to have a lower risk for infection (OR 0.7, 95% CI 0.5–0.9) and GDU pigs to have a higher risk for infection (OR 1.6, 95% CI 1.2–2.1) when compared with BTW pigs. Finally, Illinois had higher odds for IAV infection (OR 3.2, 95% CI 2.6–4.0) compared with the other 3 states.

IAV subtypes were determined for 25.2% of the IAV positive samples (Figure 2). All 3 common porcine influenza subtypes (H1N1, H1N2, and H3N2) were detected

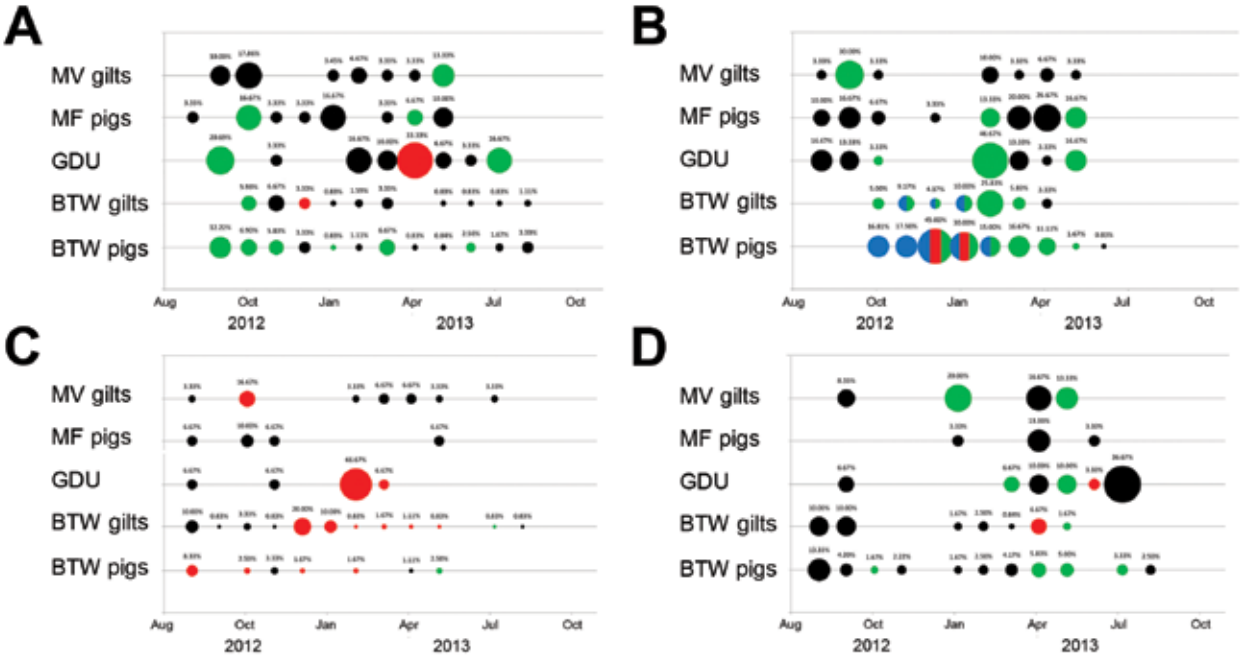


**Figure 2.** Prevalence of influenza A virus by system, farm type, and month, United States, September 2012–September 2013. In A: system 1; B) system 2; C) system 3; and D) system 4, positive RNA nasal swab samples were categorized by age range (gilt, female pig 5–6 months of age or piglet, ≈3 weeks of age) and production point farm (multiplier farms [MF] Gilt, MF Pig, GDU, BTW Gilt, BTW Pig). Each circle depicts a month during which positive influenza samples were collected; percentage of positive swabs is listed above each circle. Influenza A subtypes are indicated by circle color: green, H1N1; blue, H1N2; red, H3N2; black, untyped. Multicolored circles indicate the detection of >1 subtype.

**Table 1.** Epidemiologic data for influenza A virus among swine in coordinated swine production systems, United States, September 2012–September 2013*

| Variable | No. samples | No. (%) positive samples | p value |
|---|---|---|---|
| State* | | | |
| Georgia | 2,520 | 73 (2.9) | |
| Illinois | 4,490 | 408 (9.1) | <0.001 |
| Nebraska | 3,894 | 126 (3.2) | |
| Oklahoma | 4,050 | 134 (3.3) | |
| Age group | | | |
| Gilt | 8,028 | 375 (4.7) | 0.85 |
| Piglet | 6,926 | 366 (5.3) | |
| Farm type | | | |
| MF, gilts† | 1,526 | 67 (4.4) | |
| MF, piglets | 1,559 | 72 (4.6) | |
| GDU | 1,455 | 115 (7.9) | <0.001 |
| BTW | 10,414 | 487 (4.7) | |
| System | | | |
| 1 | 3,673 | 142 (3.9) | |
| 2 | 3,337 | 339 (10.2) | <0.001 |
| 3 | 4,050 | 134 (3.3) | |
| 4 | 3,894 | 126 (3.2) | |

*Piglets were sampled before weaning; gilts were sampled at entry to MFs and before moving from GDUs to BTWs. MF, multiplier farm; GDU, gilt development unit; BTW, breed-to-wean.

during the 13-month surveillance period. Increased prevalence of IAV was detected in piglets (MF pigs, GDU, BTW pigs) in all 4 systems, particularly in systems 1, 2, and 4, from winter to early summer (Figure 2, panels A, B, D), which is consistent with other studies (*8*). Multiple subtype detection occurred only on BTW farms in system 2 (Figure 2, panel B).

## Conclusions

We found that IAV infection was present at all stages of swine production within coordinated production systems. Animal age, geographic location, and type of farm affected risk for infection. We also found continuing virus circulation in all populations year round, although prevalence was higher from winter through early summer. To fully elucidate the factors that contribute to persistent IAV infection in swine farms and therefore develop evidence-based control strategies, further research is needed.

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**Table 2.** Correlation between age, state, farm type or production system, and influenza A virus status in swine in coordinated production systems, United States, September 2012–September 2013*

| Factor | aOR (95% CI) | p value |
|---|---|---|
| Age group | | |
| Piglet vs. gilt | 1.3 (1.1–1.6) | 0.004 |
| State | | |
| Illinois vs. Oklahoma | 1.9 (1.4–2.6) | <0.001 |
| Farm type | | |
| MF piglets vs. BTW | 0.7 (0.5–0.9) | 0.011 |
| GDU vs. BTW | 1.6 (1.2–2.1) | <0.001 |
| System number | | |
| 2 vs. 1 | 1.7 (1.3–2.3) | <0.001 |

*Only statistically significant factors are shown; full analysis is provided in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/10/14-0633-Techapp1.pdf). aOR, adjusted odds ratio; CI, confidence interval; MF, multiplier farm; GDU, gilt development unit; BTW, breed-to-wean.

Dr. Kaplan is a postdoctoral research associate at St. Jude Children's Research Hospital in Memphis. His research interests include virus ecology, virus evolution, and the molecular determinants of virus host range.

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# Cross-sectional Serosurvey of Crimean-Congo Hemorrhagic Fever Virus IgG in Livestock, India, 2013–2014

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We conducted a cross-sectional serosurvey of Crimean-Congo hemorrhagic fever (CCHF) among livestock in 22 states and 1 union territory of India. A total of 5,636 samples from bovines, sheep, and goats were screened for CCHF virus IgG. IgG was detected in 354 samples, indicating that this virus is widespread in this country.

Crimean-Congo hemorrhagic fever (CCHF) is caused by a virus (CCHFV) that belongs to the family *Bunyaviridae*, genus *Nairovirus* (*1,2*). CCHF causes severe illness in humans and has a case-fatality rate of up to 80% (*3,4*). The disease is widespread in various countries in Africa, Asia, southeastern Europe, and Eurasia, and cases have been documented recently in India (*4–7*). The virus is transmitted to humans when they are bitten by *Hyalomma* spp. ticks, which are usually found on cattle, buffalo, goats, and sheep. Humans can also be infected through nosocomial transmission or from the blood, tissues, or bodily secretions of an infected animal when it is slaughtered or during related procedures (*1,4,8,9*).

The first confirmed cases of CCHF in India occurred during a nosocomial outbreak in Ahmadabad, Gujarat, in January 2011 (*9*). During 2012–2015, several outbreaks and cases of CCHF transmitted by ticks via livestock and several nosocomial infections were reported in the states of Gujarat and Rajasthan. Cases were documented from 6 districts of Gujarat (Ahmadabad, Amreli, Patan, Surendranagar, Kutch, and Aravalli) and 3 districts of Rajasthan (Sirohi, Jodhpur, and Jaisalmer) (*10,11*). Recently, a CCHF case was reported from Uttar Pradesh state (http://promedmail.chip.org/pipermail/promed-soas/2015-February/000582.html).

In the past, serologic evidence of CCHFV infection was reported in India from animal serum samples collected in western border districts, southern regions, Maharashtra state, and Jammu and Kashmir state (*12*). A recent serosurvey conducted in 15 districts of Gujarat revealed the presence of CCHFV IgG in a substantial proportion of domestic animals (*13*). On the basis of these data, we conducted a countrywide cross-sectional serosurvey of livestock to determine the presence of CCHFV in India.

## The Study

Working with the Indian Council of Agricultural Research, we asked foot and mouth disease (FMD) centers throughout India to send us serum samples from bovines, goats, and sheep. We requested ≥200 representative samples from each state and only used those that tested negative for FMD. The number of samples varied (99–357 for bovine samples and 19–260 for sheep and goat samples), depending on where the samples were collected and the population of each animal in that area.

We detected CCHFV-specific IgG in the serum samples by using 2 ELISA kits (1 for bovines and 1 for sheep and goats) that were developed by the National Institute of Virology (NIV) in Pune, India. We coated Nunc MaxiSorp plates (Thermo Fisher Scientific, Waltham, MA, USA) with γ-inactivated CCHFV (positive antigen) and negative control tissue culture fluid (negative antigen) diluted in carbonate buffer and incubated them overnight at 4°C. Plates were washed 3 times with 1× phosphate-buffered saline with 1% Tween-20 (PBST) and further treated with postcoating buffer. Plates were washed then 3 times with 1× PBST. Serum samples were diluted in sample dilution buffer (1:200 dilutions for bovine samples and 1:2,000 dilutions for sheep/goat samples). Positive and negative control animal serum samples were included in triplicate for each assay by using similar dilution for quality control.

Samples were added to both the positive and negative antigen-coated rows and incubated at 37°C for 45 min. After washing the plates 5 times with 1× PBST, we probed the wells with bovine or sheep IgG conjugated with biotin for the respective ELISAs and incubated the plates for 1 hour. We washed the plates 5 times with 1× PBST, incubated them with avidin-horseradish peroxidase for 30 min at

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37°C, then washed them 5 times with 1× PBST. We added 3,3′,5,5′-tetramethylbenzidine substrate and incubated the plates for 10 min in the dark at room temperature; the reaction was stopped by using 1N $H_2SO_4$. Finally, we read the plates with a spectrophotometer at 450 nm. The ratio of optical density of positive and negative controls was taken for each sample (P/N ratio). The sample was considered positive when the P/N ratio was >1.5 from both kits (*14*).

The sensitivity and specificity of these kits were tested and compared with known standards by using ELISA reagents provided by the Centers for Disease Control and Prevention (Atlanta, GA, USA). Bovine ELISA showed 80.5% sensitivity and 96.05% specificity, and sheep/goat ELISA showed 63.6% sensitivity and 100.0% specificity. The performance of these kits was validated by 3 NIV laboratories and 3 other national laboratories in India.

We screened 5,636 (4,781 bovine and 855 sheep and goat) animal serum samples from 22 states and 1 union territory for CCHFV IgG; samples were not obtained from 7 states and 6 union territories. Only 8 states and 1 union territory provided sheep and goat samples. Overall, 260 (5.43%) of 4,781 bovine samples and 94 (10.99%) of 855 sheep/goat samples tested positive for CCHFV IgG (Table; Figure). Among bovine samples, maximum IgG positivity was seen in Orissa (31.3%); for sheep and goat samples, maximum IgG positivity was seen in Himachal Pradesh (53.1%) (Table). Seropositivity levels found in these animals suggested prevalence of CCHFV infection among livestock in these states across the country.


**Figure**. Location of Crimean-Congo hemorrhagic fever virus IgG seropositivity in bovines, sheep, and goats in 22 states and 1 union territory, India. Gray shading, seropositivity in bovines; black dots, seropositivity in sheep/goats; white, serum samples not available screening.

**Table.** CCHFV IgG positivity detected in serum samples from bovines, sheep, and goats from 22 states and 1 union territory, India*

| Location of center | No. positive samples/no. samples tested (%) | |
|---|---|---|
| | Bovine | Sheep and goat |
| Andhra Pradesh | 30/233 (12.9) | – |
| Madhya Pradesh | 7/150 (4.7) | 3/40 (7.5) |
| Maharashtra | 16/240 (6.7) | – |
| Punjab | 6/198 (3.0) | – |
| Rajasthan | 31/295 (10.5) | – |
| Orissa | 31/99 (31.3) | – |
| Arunachal Pradesh | 13/150 (8.7) | 5/71 (7.0) |
| Karnataka | 15/219 (6.8) | – |
| Kerala | 5/200 (2.5) | 5/219 (2.3) |
| West Bengal | 13/357 (3.6) | – |
| Manipur | 1/146 (0.7) | 0/49 |
| Mizoram | 15/203 (7.4) | – |
| Tamil Nadu | 6/379 (1.6) | – |
| Uttar Pradesh | 7/195 (3.6) | – |
| Tripura | 1/149 (0.7) | – |
| Guwahati | 26/149 (17.0) | 0/50 |
| Haryana | 3/200 (1.5) | – |
| Himachal Pradesh | 1/150 (0.7) | 26/49 (53.1) |
| Jammu and Kashmir | 4/182 (2.2) | 0/19 |
| Nagaland | 8/199 (4.0) | – |
| Andaman and Nicobar | 2/308 (0.7) | 21/98 (21.4) |
| Uttarakhand | 2/100 (2.0) | – |
| Gujarat | 17/280 (6.1) | 34/260 (13.1) |
| Total | 260/4,781 (5.43) | 94/855 (10.99) |

*CCHFV, Crimean-Congo hemorrhagic fever virus; FMD, foot and mouth disease; –, no samples available for screening.

## Conclusions

A large portion of the economy of India and the country's rural development depend on agriculture, livestock farming, and the dairy industry (http://poshan.nic.in/jspui/bitstream/DL/1247/1/nfi_01_00_643.pdf, http://dahd.nic.in/dahd/updates/whats-new/18th-livestock-census-2007.aspx). Because India hosts many animal trading fairs each year (e.g., Pushkar fair, Uttar Pradesh; Sonepur Animal Mela, Bihar), tick-infested animals move throughout the country. India also exports >US$400 million of meat. Such widespread animal trade and exports can pose a high threat of transmission of pathogens such as CCHFV to newer areas. The country experienced similar situations during suspected plague outbreaks and outbreaks of infection with avian influenza, which not only resulted in considerable economic losses but also created panic in the community. Although our survey showed scattered geographic distribution of CCHFV IgG among livestock in India, data from 5 years of investigations in Gujarat suggest that active surveillance in any of these states would probably reveal a more accurate estimate of CCHF prevalence.

This study suggests that animal husbandry and abattoir workers are at high risk because they are always in close contact with livestock or carcasses that may be infested with CCHFV-infected ticks (Table; Figure). Because viremia in livestock is short-lived (up to 2 weeks) and of low intensity, infected animals do not develop severe disease, but they may still transmit the virus to other animals and to humans.

Diagnosis of high-risk group pathogens is a major concern in India, where few Biosafety Level 3 laboratories and only 1 Biosafety Level 4 laboratory exist. Therefore, there is also a need to make available safe diagnostic tests that can be used at primary health centers, medical colleges, and all other health settings across the country. The CCHFV IgG ELISA kits developed by NIV could help in monitoring CCHFV prevalence and the findings could make it possible for public health authorities to develop proactive preparedness programs that would enable them to send alerts and develop precautionary measures.

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# Novel Paramyxoviruses in Bats from Sub-Saharan Africa, 2007–2012

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As part of a larger survey for detection of pathogens among wildlife in sub-Saharan Africa conducted during 2007–2012, multiple diverse paramyxovirus sequences were detected in renal tissues of bats. Phylogenetic analysis supports the presence of at least 2 major viral lineages and suggests that paramyxoviruses are strongly associated with several bat genera.

Members of the *Paramyxoviridae* family are enveloped negative-sense RNA viruses, further classified into either the *Pneumovirinae* or *Paramyxovirinae* subfamily (*1*).The *Paramyxovirinae* subfamily has increasingly been associated with bat species across the globe. The *Henipavirus* genus is 1 of 7 genera in this subfamily and contains the first recorded zoonotic paramyxoviruses, Hendra virus and Nipah virus. These 2 viruses are associated with severe respiratory and neurologic syndromes, and regular spillover from *Pteropus* spp. bats causes infections in humans and domestic animals (*2*).

Enhanced surveillance for bat-associated pathogens has led to the discovery of numerous novel paramyxoviruses (*3–5*). *Henipavirus*-related viruses were identified in another pteropodid species, *Eidolon helvum*, sampled in Ghana, West Africa. This finding suggests an extension of the geographic and host ranges of the members of this virus genus (*6*). Subsequent studies demonstrated a high diversity of paramyxoviruses in *E. helvum* bat population in Africa, as well as in other bat species from different continents. This finding suggests that bats may have a global role as potential paramyxovirus reservoirs (*3,4*). To contribute toward the knowledge of bat-associated paramyxovirus diversity and distribution, we sampled multiple bat species from several sub-Saharan African countries.

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## The Study

During 2007–2012, we sampled 1,220 bats representing at least 48 species from multiple locations in selected countries in Africa (Table 1). Bats were anesthetized with the use of ketamine (0.05–0.1 mg/g body mass) and exsanguinated by cardiac puncture. Voucher specimens were identified through morphologic characterization (*7*) or, alternatively, through genetic barcoding. Approximately 30–100 mg of renal tissue was used for RNA extraction. A heminested primer set targeting the conserved polymerase (large) gene of *Respirovirus, Morbillivirus,* and *Henipavirus* was used for sample screening through reverse transcription PCR (*8*). A total of 103 samples (8.4%) tested positive, and the obtained amplicons of ≈490 bp were sequenced (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/10/14-0368-Techapp1.pdf). For phylogenetic analysis, representative paramyxovirus sequences available from GenBank were included (online Technical Appendix Table 2), and Bayesian analysis was performed by using BEAST version 1.7.4 software (http://beast.bio.ed.ac.uk/) (Figure; http://wwwnc.cdc.gov/EID/article/21/10/14-1368-F1.htm).

Several samples from bat species not previously implicated as paramyxovirus reservoirs tested positive in our study. Some of these implicated species are known to roost in peridomestic environments. Sequence analysis of paramyxovirus sequences showed a clear bifurcation of the phylogenetic tree, segregating paramyxoviruses detected in pteropodid bats (Pteropodidae) from paramyxoviruses detected in bats of other families (Figure). The former contained henipaviruses and related viruses. Two viral sequences detected in *Rousettus aegyptiacus* bats grouped within this cluster as part of a sister clade to the henipaviruses. The second cluster contained sequences derived from nonpteropodid bats. Some of these sequences grouped with the sequences from the *Morbillivirus* and proposed *Jeilongvirus* genera, whereas others could not be included in any of the other paramyxovirus genera.

We observed a strong association of several viral lineages to particular bat genera for paramyxoviruses identified in *Hipposideros*, *Miniopterus*, *Coleura*, *Myotis,* and *Pipistrellus* bats, although the bats were sampled from geographically distant locations. In contrast to the sequences of

**Table 1.** African bat species sampled and the number of paramyxovirus sequences detected in sub-Saharan Africa, by country, 2007–2012*

| | | |
|---|---|---|
| **Southern Africa** | | |
| South Africa | | |
| *Chaerephon ansorgei* (2/0) | ***Neoromicia nana* (7/2)** | *Rhinolophus* sp. (1/0) |
| *Chaerephon pumilus* (8/0) | *Neoromicia rueppellii* (1/0) | *Rousettus aegyptiacus* (18/0) |
| *Epomophorus gambianus* (2/0) | *Neoromicia zuluensis* (1/0) | *Sauromys petrophilus* (1/0) |
| *Epomophorus wahlbergi* (15/0) | ***Nycteris thebaica* (12/1)** | *Scotophilus* sp. (12/0) |
| ***Eptesicus hottentotus* (2/1)** | *Nycticeinops schlieffeni* (9/0) | *Scotophilus dinganii* (26/0) |
| *Glauconycteris variegata* (5/0) | *Pipistrellus hesperidus* (5/0) | *Scotophilus leucogaster* (2/0) |
| ***Hipposideros caffer* (6/2)** | *Pipistrellus rusticus* (5/0) | *Scotophilus nigrita* (1/0) |
| ***Kerivoula argentata* (1/1)** | *Pipistrellus* sp. (5/0) | *Scotophilus viridis* (3/0) |
| *Miniopterus natalensis* (5/0) | *Rhinolophus darlingi* (5/0) | *Tadarida aegyptiaca* (5/0) |
| *Miniopterus* sp. (37/0) | ***Rhinolophus denti* (3/2)** | *Taphozous mauritianus* (2/0) |
| *Mops condylurus* (7/0) | *Rhinolophus fumigatus* (2/0) | |
| *Neoromicia capensis* (16/0) | ***Rhinolophus landeri* (1/1)** | |
| *Neoromicia helios* (6/0) | *Rhinolophus simulator* (2/0) | |
| Swaziland | | |
| *Nycteris thebaica* (4/0) | | |
| **Eastern Africa** | | |
| Kenya | | |
| *Coleura afra* (27/10) | *Miniopterus natalensis* (15/0) | ***Rousettus aegyptiacus (84/2)*** |
| *Eidolon helvum* (15/0) | ***Miniopterus* sp. (77/13)** | *Scotoecus* sp. (2/0) |
| *Epomophorus labiatus* (6/0) | *Neoromicia* sp. (25/0) | *Scotophilus dinganii* (2/0) |
| *Epomophorus wahlbergi* (2/0) | ***Nycteris* sp. (2/1)** | *Taphozous* sp. (1/0) |
| *Hipposideros vittatus* (71/0) | ***Otomops martiensseni* (40/9)** | ***Triaenops afer* (16/12)** |
| ***Hipposideros* sp. (8/1)** | *Rhinolophus landeri* (12/0) | |
| ***Miniopterus minor* (151/14)** | *Rhinolophus* sp. (14/0) | |
| **Central Africa** | | |
| Cameroon | | |
| *Chaerephon* sp. (32/0) | ***Hipposideros* sp. (39/1)** | ***Taphozous* sp. (12/3)** |
| *Eidolon helvum* (15/0) | ***Rhinolophus* sp. (9/1)** | |
| *Epomophorus* sp. (1/0) | *Scotophilus dinganii* (1/0) | |
| Democratic Republic of the Congo | | |
| *Chaerephon pumilus* (25/0) | *Hypsignathus monstrosus* (2/0) | *Myonycteris torquata* (8/0) |
| *Chaerephon* sp. (22/0) | *Megaloglossus woermanni* (10/0) | *Myotis* sp. (3/0) |
| *Eidolon helvum* (22/0) | *Micropteropus pusillus* (1/0) | *Neoromicia* sp. (1/0) |
| *Glauconycteris argentata* (1/0) | *Mimetillus moloneyi* (1/0) | ***Pipistrellus* sp. (40/20)** |
| ***Hipposideros fuliginosus* (21/3)** | ***Miniopterus* sp. (41/2)** | *Rhinolophus* sp. (1/0) |
| *Hipposideros gigas* (2/0) | *Mops condylurus* (33/0) | *Scotophilus dinganii* (2/0) |
| **Western Africa** | | |
| Nigeria | | |
| *Eidolon helvum* (20/0) | ***Hipposideros* sp. (3/1)** | *Rousettus aegyptiacus* (21/0) |
| *Hipposideros vittatus* (8/0) | *Lissonycteris angolensis* (8/0) | |

*Values are no. samples (no. positive). Boldface indicates implicated species. The sampling protocol was approved by the Institutional Animal Care and Use Committee of the Centers for Disease Control and Prevantion; protocol 2096FRAMULX-A3 and The University of Pretoria Animal Ethics Committee (EC054–14).

European and South American origin, for which geographic clustering was observed, no such clustering was found among the sequences from African bats.

The incidence and diversity of viral sequences varied according to bat species. For example, nearly identical sequences were detected in 50% of *Pipistrellus* spp. sampled from a single colony in the Democratic Republic of the Congo (n = 40). In other cases, several distinct viral sequences were detected in different individual bats of 1 species, such as *Miniopterus minor* bats sampled from a single colony in Kenya (n = 53), which harbored 6 distinct viral sequences. Some of the sequences were found more frequently than others. In contrast to a previous study which did not identify paramyxoviruses in *Coleura afra* bats sampled in Ghana (n = 71) (*4*), we detected a substantial paramyxovirus incidence (37%, n = 27) in the same bat species sampled in Kenya (Table 2).

## Conclusions

The henipaviruses were the first bat paramyxoviruses directly linked to human disease; however, most aspects of pathogenicity and the host ranges of the increasingly detected novel bat paramyxoviruses remain to be investigated. Here we report information regarding paramyxovirus distribution through molecular evidence of bat-associated paramyxoviruses in Cameroon, Nigeria, and South Africa, as well as evidence of paramyxoviruses in nonpteropodid bats from the Democratic Republic of the Congo. Our results suggest that 2 separate lineages were established during the evolution of bat-associated paramyxoviruses:

**Table 2**. Paramyxovirus incidence in selected bat species from various African countries*

| Species | Country | Tissue type† | No. sampled | No. positive | Incidence, % | Reference |
|---|---|---|---|---|---|---|
| *Coleura afra* | Ghana | ‡ | 71 | 0 | 0.0 | (*4*) |
| | Kenya | Kidney | 27 | 10 | 37.0 | § |
| | Central Africa¶ | Spleen | 25 | 1 | 4.0 | (*4*) |
| *Eidolon helvum* | Cameroon | Kidney | 15 | 0 | 0.0 | § |
| | DRC | Kidney | 22 | 0 | 0.0 | § |
| | Ghana | All solid organs, blood | 673 | 67 | 10.0 | (*4*) |
| | Kenya | Kidney | 15 | 0 | 0.0 | § |
| | Central Africa | Spleen | 49 | 17 | 34.5 | (*4*) |
| | Nigeria | Kidney | 20 | 0 | 0.0 | § |
| | Republic of Congo | All solid organs, blood, salivary gland, throat swab, feces, urine | 42 | 11 | 26.2 | (*9*) |
| *Epomophorus gambianus* | Central Africa | Spleen | 48 | 3 | 6.3 | (*4*) |
| | South Africa | Kidney | 2 | 0 | 0.0 | § |
| | Ghana | ‡ | 20 | 1 | 5.0 | (*4*) |
| *Hipposideros caffer* | Central Africa | Spleen | 337 | 3 | 0.9 | (*4*) |
| | South Africa | Kidney | 6 | 2 | 33.3 | § |
| | DRC | Kidney | 2 | 0 | 0.0 | § |
| *Hipposideros gigas* | Gabon | Spleen | 196 | 3 | 1.5 | (*4*) |
| | DRC | Kidney | 2 | 0 | 0.0 | § |
| *Hypsignathus monstrosus* | Central Africa | Spleen | 53 | 4 | 7.5 | (*4*) |
| | DRC | Kidney | 10 | 0 | 0.0 | § |
| *Megaloglossus woermanni* | Central Africa | Spleen | 34 | 1 | 2.9 | (*4*) |
| | DRC | Kidney | 8 | 0 | 0.0 | § |
| *Myonycteris torquata* | Central Africa | Spleen | 111 | 3 | 2.7 | (*4*) |
| | Ghana | ‡ | 1 | 0 | 0.0 | (*4*) |
| *Rhinolophus landeri* | Kenya | Kidney | 12 | 0 | 0.0 | § |
| | South Africa | Kidney | 1 | 1 | 100.0 | § |
| | Ghana | ‡ | 30 | 0 | 0.0 | (*4*) |
| *Rousettus aegyptiacus* | Kenya | Kidney | 84 | 2 | 2.4 | § |
| | Central Africa | Spleen | 183 | 18 | 9.8 | (*4*) |
| | Nigeria | Kidney | 21 | 0 | 0.0 | § |
| | South Africa | Kidney | 18 | 0 | 0.0 | § |

*DRC, Democratic Republic of the Congo.
†Tissue type stated for positive samples only and may not indicate all tissues sampled.
‡Information not available.
§Species and countries sampled during this study.
¶Central Africa refers to Gabon/Republic of Congo/DRC/Republic of Central Africa.

the pteropodid bats potentially harbor 1 lineage, and the nonpteropodid bats potentially harbor the other. In contrast to the proposed chiropteran classification, which supports a sister-taxon relationship between Rhinolophoidae and Pteropodidae on the suborder level, paramyxovirus divergence appears to correlate with traditional bat taxonomy. The evolution behind this divergence might be a result of multiple evolutionary origins or a single origin with subsequent divergence. As with the evolution of echolocation, this question remains to be answered (*11*). More extensive bat sampling and molecular dating of the paramyxovirus phylogeny may help resolve this question.

Intensified anthropogenic transformations have facilitated closer contact between humans, domestic animal populations, and wildlife. Our study demonstrates that some bat species, adapted to peridomestic roosting, can have a substantial incidence of diverse paramyxoviruses. The variation in incidence and viral diversity observed in several bat species may suggest that some species are the true reservoirs, whereas others are mere incidental hosts. Given the observed virus diversity, implications for public health and veterinary medicine should be taken into account, especially considering the known likelihood of direct bat-to-human and human-to-human transmission of Nipah virus (*12*). Enhanced surveillance in bats and other animals will be useful for detecting possible spillover events and host shifts. Clearly, systematic longitudinal studies are needed to elucidate critical factors of paramyxovirus circulation within bat communities (*13*), and further research is needed to clarify the pathobiology, tissue tropism, and excretion pathways of these novel paramyxoviruses because these factors can be directly related to their zoonotic potential.

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# Methicillin-Susceptible, Vancomycin-Resistant *Staphylococcus aureus*, Brazil

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We report characterization of a methicillin-susceptible, vancomycin-resistant bloodstream isolate of *Staphylococcus aureus* recovered from a patient in Brazil. Emergence of vancomycin resistance in methicillin-susceptible *S. aureus* would indicate that this resistance trait might be poised to disseminate more rapidly among *S. aureus* and represents a major public health threat.

Acquisition of high-level vancomycin resistance by *Staphylococcus aureus* represents a major public health risk because this antimicrobial drug continues to be the first-line and most inexpensive therapy to treat methicillin-resistant *S. aureus* (MRSA) despite concerns about its clinical efficacy. Recently, we described vancomycin-resistant MRSA (VR-MRSA) recovered from the bloodstream of a patient in Brazil (*1*). VR-MRSA belongs to sequence type (ST) 8 and is phylogenetically related to the community-associated (CA) MRSA USA300 genetic lineage that has rapidly disseminated in the United States and the northern region of South America (USA300-Latin American variant [USA300-LV]) (*1,2*). The *vanA* gene cluster in VR-MRSA was carried by a transferable staphylococcal plasmid (pBRZ01). We characterized a clinical isolate of vancomycin-resistant, methicillin-susceptible *S. aureus* (VR-MSSA) and document the in vivo transfer of the *vanA* gene cluster to 2 unrelated *S. aureus* strains causing bacteremia within the same patient.

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## The Study

On August 28, 2012, a blood culture from a patient in Brazil was reported positive for 2 isolates of MSSA while the patient was receiving daptomycin therapy (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/14-1914-Techapp1.pdf). One MSSA isolate was susceptible to all antimicrobial drugs tested (VS-MSSA). The second isolate (VR-MSSA) had a vancomycin MIC of 256 μg/mL and was also resistant to gentamicin (Table 1). Both isolates were susceptible to daptomycin (MIC 0.5 μg/mL). Thirteen days earlier, 2 MRSA isolates, 1 of which was resistant to vancomycin (VR-MRSA), were recovered from the blood of the same patient (online Technical Appendix) (*1*). The daptomycin MICs for both MRSA strains were also 0.5 μg/mL.

Bacterial strains used in this study (Table 1) were grown in brain–heart infusion broth and agar. Plasmid pBRZ01 was transferred by using filter mating (*3*) and VR-MSSA and VR-MRSA as donors and VS-MSSA, VS-MRSA, and RN4220RF as recipients (Table 1). Transconjugants were selected on brain heart infusion medium containing vancomycin (32 μg/mL) and fusidic acid (25 μg/mL). Colonies from each mating experiment were subjected to digestion with *Sma*I and pulsed-field gel electrophoresis to investigate genetic relatedness (*1*). Plasmids carrying the *vanA* gene cluster were detected by using S1 nuclease digestion followed by hybridization with a *vanA* probe (*4*).

Whole-genome sequencing of VR-MSSA, VS-MSSA, and 2 representatives of the Chilean/Cordobes clone (M1, M91) was performed by using MiSeq PacBio RS II (Illumina, San Diego, CA, USA) to close the VR-MSSA genome (*5*) (online Technical Appendix). Phylogenetic analysis was performed by using the maximum-likelihood framework within RAxML v7.4.2 (*6*). For cell wall analysis, extraction and separation of peptidoglycan precursors was performed as described (*7*).

The PFGE patterns of both isolates (VR-MSSA and VS-MSSA) were indistinguishable, and in vitro growth rates were similar (Figure 1, panel A). S1 nuclease analyses indicated that VR-MSSA harbored a plasmid of ≈55

**Table 1.** *Staphylococcus aureus* strains used in analysis of methicillin and vancomycin resistance, Brazil*

| Strain | Strain characteristics | MIC, μg/mL | | Reference |
|---|---|---|---|---|
| | | Vancomycin | Gentamicin | |
| VS-MRSA | Isolated from the bloodstream of a patient in Brazil | 0.5 | 0.5 | (*1*) |
| VR-MRSA | Isolated from the blood of the same patient above and carrying *vanA*-containing pBRZ01 | >256 | 32 | (*1*) |
| VS-MSSA | Isolated from the blood of the same patient 13 d after isolation of VR-MRSA | 1 | 0.75 | This study |
| VR-MSSA | Isolated from the same blood culture as VS-MSSA | 256 | 48 | This study |
| RN4220-RF | Laboratory strain of *S.aureus* used as recipient for mating experiments; fusidic acid and rifampin-resistant | 1 | 1 | (*1*) |
| Transconjugant 1† | Transconjugant obtained from a mating experiment using VR-MSSA as donor and VS-MRSA as recipient | >256 | 48 | This study |
| Transconjugant 2† | Transconjugant obtained from a mating experiment using VR-MRSA as donor and VS-MSSA as recipient | >256 | 64 | This study |
| Transconjugant 3† | Transconjugant obtained from a mating experiment using VR-MSSA as donor and RN4220-RF as recipient | >256 | 64 | This study |

*VS-MRSA, vancomycin-susceptible, methicillin-resistant *S. aureus*; VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA, vancomycin-resistant, methicillin-susceptible *S. aureus*.
†A fusidic acid–resistant derivative was generated for mating experiments. All mating experiments were performed on brain heart infusion agar in the presence of vancomycin (32 μg/mL) and fusidic acid (25 μg/mL) to select for transconjugants.

kb, which yielded a positive result when hybridized with a *vanA* probe (Figure 1, panels B, C) and was similar in size to the previously described *vanA*-containing plasmid pBRZ01 identified in the same patient (*1*). pBRZ01 of VR-MSSA was readily transferred to *S. aureus* RN4220-RF (efficiency = $3 \times 10^{-5}$/donor). In vitro conjugative transfer of pBRZ01 between MRSA and MSSA strains recovered from the patient's bloodstream was also readily achieved with efficiencies ranging from $4.3 \times 10^{-7}$/donor to $2.5 \times 10^{-6}$/donor. Acquisition of the pBRZ01 by corresponding strains resulted in resistance to vancomycin and gentamicin (Table 1).

Genome sequencing (online Technical Appendix) showed that VR-MSSA and VS-MSSA belong to clonal complex (CC) 5 (sequence type ST5) and harbor staphylococcal protein A (Spa) type t002. VS-MSSA and VR-MSSA have the characteristic CC5 genetic traits described by Kos et al. (*8*). The genome of VR-MSSA has a 2,906,602-bp chromosome and 3 extrachromosomal elements, including a plasmid of 55,713 bp identical to the previously described *vanA*-carrying pBRZ01 (*1*), which also harbors *aac(6')-aph(2")*, which confers gentamicin resistance.

Comparison of the core genomes of VR-MSSA and VS-MSSA showed only 20 single-nucleotide polymorphism differences, which suggested a close genetic relationship and probably representing the same organism that acquired pBRZ01. Phylogenetic analysis (Figure 2) confirmed that VR-MSSA is not a derivative of VR-MRSA (*1*)

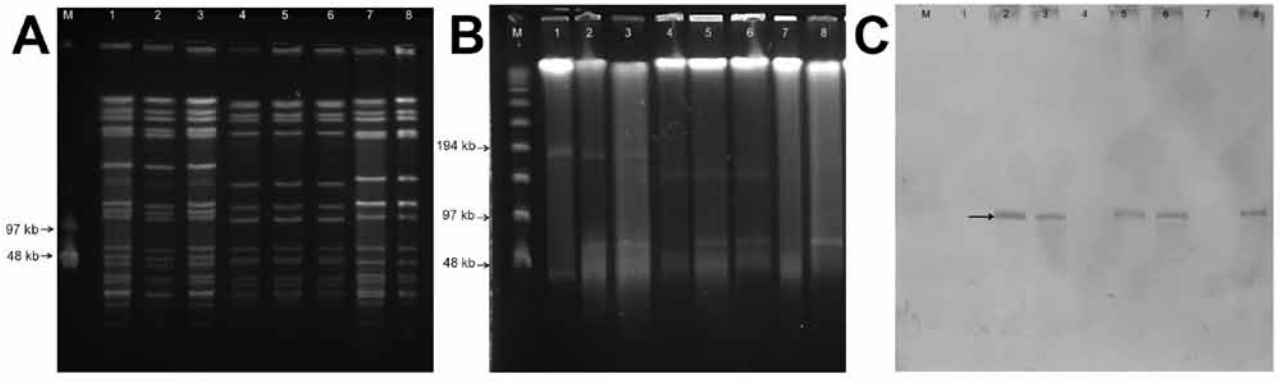


**Figure 1.** Molecular typing of *Staphylococcus aureus* strains, Brazil. A) *Sma*I digestion of total DNA, followed by pulsed-field gel electrophoresis. Lane M, lambda ladder (molecular masses are indicated in kilobases on the left); lane 1, vancomycin-susceptible, methicillin-resistant *S. aureus* (VS-MRSA) isolated from the blood of a Brazilian patient (*1*); lane 2, vancomycin-resistant MRSA (VR-MRSA) isolated from the same patient and blood culture (*1*); lane 3, transconjugant 1 obtained from a mating experiment using vancomycin-resistant MSSA (VR-MSSA) as donor and VS-MRSA as recipient; lane 4, vancomycin-susceptible MSSA (VS-MSSA) isolated from the blood of the same patient 13 days after isolation of VR-MRSA; lane 5, VR-MSSA isolated at the same time as VS-MSSA; lane 6, transconjugant 2 obtained from a mating experiment using VR-MRSA as donor and VS-MSSA as recipient; lane 7, *S. aureus* RN4220 RF, lane 8, transconjugant 3 obtained using VR-MSSA as donor and RN4220 RF as recipient. B) S1 digestion of total DNA using the same strains shown in panel A. C) Hybridization with *vanA* probe using the same strains shown in panel A. Arrow indicates a positive signal for the *vanA* gene.

(isolated days before from the same patient) and emphasized the relationship of this strain to other vancomycin-resistant *S. aureus* and MRSA isolates with intermediate susceptibility to vancomycin (VISA).

We analyzed the pool of cytoplasmic peptidoglycan precursors of VR-MSSA grown in the absence or presence of 50 μg/mL of vancomycin for induction of the *vanA* cluster (Table 2). Tandem mass spectrometry analysis identified 3 nucleotide precursors ending in D-alanyl-D-alanine (UDP-MurNAc-pentapeptide), D-alanyl-D-lactate (UDP-MurNAc-pentadepsipeptide), and D-Ala (UDP-MurNAc-tetrapeptide). Upon induction with vancomycin, UDP-MurNAc-pentapeptide was not detected, and UDP-MurNAc-pentadepsipeptide accounted for most of the precursors (Table 2). These results indicate that the *van*-encoded enzymes required for incorporation of D-Lac into the precursors were fully functional in VR-MSSA. Our results also show that the *vanA* cluster was inducible by vancomycin in the *S. aureus* host because only a small proportion of the precursors (4%) ended in D-Lac in the absence of the drug.

Analyses of cell wall muropeptides from VR-MSSA showed 2 modifications of the L-Ala$^1$-γ-D-Glu$^2$-L-Lys$^3$-D-Ala$^4$-D-Ala$^5$ stem peptide that are highly conserved in *S. aureus* strains, namely the amidation of the α-carboxyl of D-Glu$^2$ to form D-iGln$^2$ and the addition of a pentaglycine side chain on the ε-amino group of L-Lys$^3$ by the Fem amino-acyltransferases (*9*). Induction of the *vanA* gene cluster

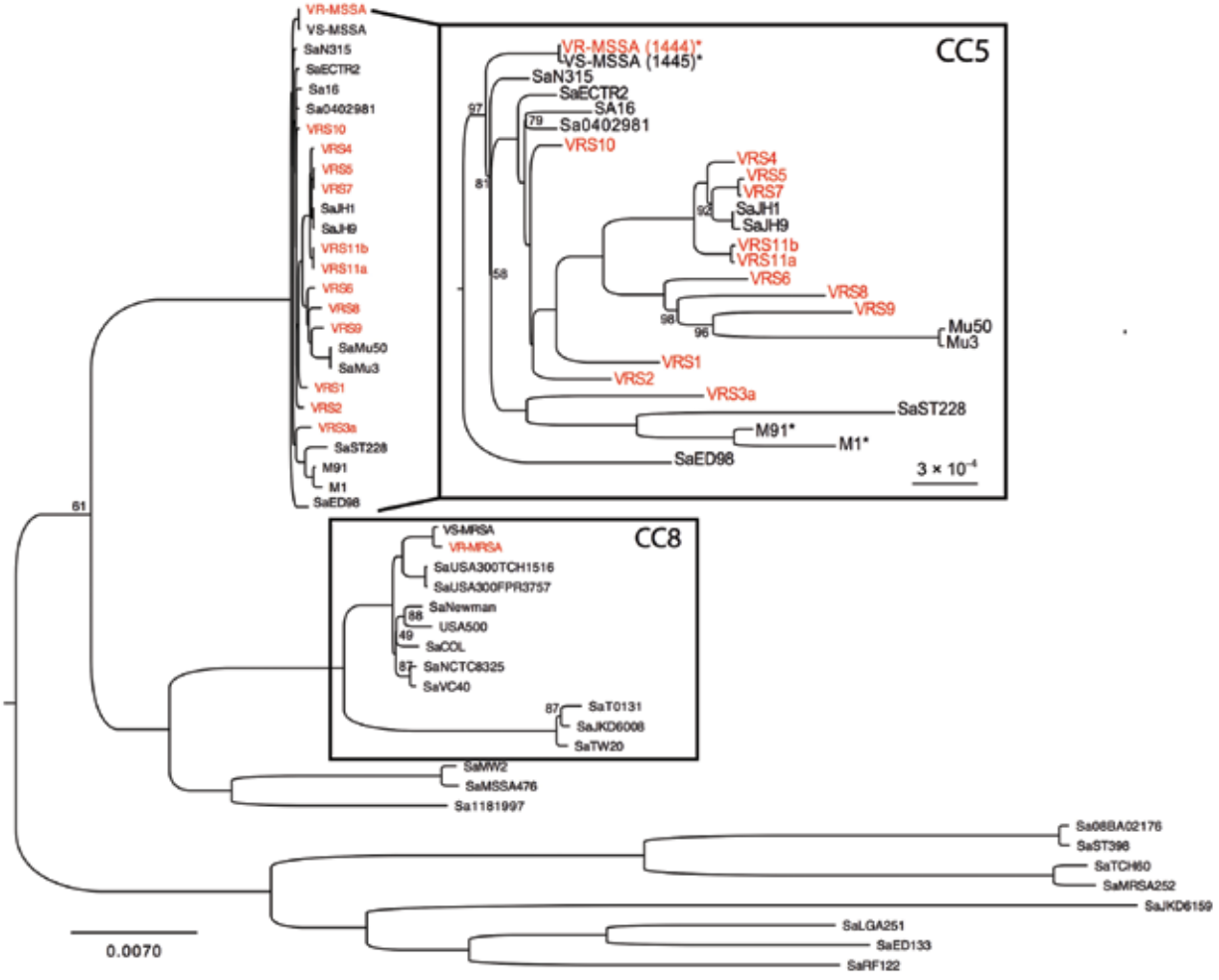


**Figure 2.** Phylogenetic analyses of *Staphylococcus aureus* strains, Brazil. Whole-genome phylogenetic tree (dataset = 325,732 single-nucleotide polymorphisms, gamma-based log likelihood – 1909607.06950) of the *S. aureus* species showing position of vancomycin-resistant, methicillin-susceptible *S. aureus* (VR-MSSA) and vancomycin-susceptible MSSA (VS-MSSA) isolates sequenced for this study. Vancomycin-resistant S. *aureus* (VRSA) strains are shown in red. Numbers on branches are bootstrap values based on 1,000 resampling iterations. All branches without numbers had bootstrap values of 100%. Branch lengths are proportional to number of nucleotide substitutions per site (scale bars). Inset labeled CC5 is expanded to emphasize the polyphyly of VRSA strains. *Genomes sequenced for this study. M1 and M91 are members of the Chilean/Cordobes clone that is widespread in Latin America (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/14-1914-Techapp1.pdf). CC, clonal complex.

**Table 2.** Relative abundance of peptidoglycan precursors in *Staphylococcus aureus* strains, Brazil*

| Precursor | Monoisotopic mass | | Abundance (%) in corresponding strains | | |
|---|---|---|---|---|---|
| | Observed | Calculated | VS-MSSA | VR-MSSA (not induced) | VR-MSSA (induced) |
| UDP-MurNaAc-tetrapeptide | 1,078.35 | 1,078.35 | 27 | 32 | 29 |
| UDP-MurNAc-pentapeptide | 1,149.37 | 1,149.39 | 73 | 64 | ND |
| UDP-MurNAc-pentadepsipeptide | 1,150.37 | 1,150.37 | ND | 4 | 71 |

*Bacteria were grown on brain heart infusion (BHI) broth (not induced) or BHI supplemented with vancomycin (50 μg/mL). VS-MSSA, vancomycin-susceptible, methicillin-susceptible *S. aureus*; VR-MSSA, vancomycin-resistant, methicillin-susceptible *S. aureus;* ND, not detected.

led to 2 major modifications. First, stem peptides ended in D-Ala$^4$, indicating that the peptidyl- D-Ala$^4$- D-Ala$^5$ target of vancomycin, and D-Ala$^4$-D-Lac$^5$ termini, were fully eliminated. Second, the pentaglycine side chain was frequently missing (online Technical Appendix), indicating that replacement of D-Ala by D-Lac at the extremity of peptidoglycan precursors might have impaired the ability of Fem transferases to add Gly on L-Lys$^3$.

## Conclusions

In this study, we demonstrated that the *vanA*-containing pBRZ01 plasmid previously described in MRSA was acquired by an invasive MSSA isolate within the same patient. Our findings also suggest that a *vanA*-containing plasmid (pBRZ01) was horizontally acquired at least twice during a short period by distinct *S. aureus* lineages within the same host (MRSA belonging to ST8 and an ST5 MSSA). VR-MSSA belongs to the ST5 lineage of CC5, a major hospital-associated lineage (*10*). The prevalent hospital-associated lineages circulating in Brazil are ST5 (New York/Japan and Pediatric clones), ST239 (Brazilian clone) and ST1 (USA400 clone) (*11*), and recent epidemiologic data showed replacement of the endemic Brazilian (ST239) clone by ST5 strains (*11–13*). Moreover, VR-MSSA is related to ST5 vancomycin-resistant *S. aureus* strains recovered in the United States (*8*) and to VISA isolates, including Mu50 and the hetero-VISA strain Mu3, initially recovered in Japan (*14*). It remains unclear why CC5 strains appear more likely to exhibit vancomycin resistance.

Our biochemical analysis indicates that the *vanA* gene cluster is fully functional in VR-MSSA, which leads to vancomycin-inducible production of D-Lac ending precursors and elimination of D-Ala- D-Ala containing peptidoglycan, as found in the enterococci (*15*). Our results also revealed a defect in side chain synthesis, although this did not prevent the synthesis of a functional and highly cross-linked peptidoglycan in VR-MSSA.

In summary, we report the in vivo acquisition of high-level vancomycin resistance in a bloodstream MSSA isolate. Of note, *vanA*-containing pBRZ01 was maintained even after the selective pressure of vancomycin had been removed, raising serious concerns about the possibility of further spread of resistance to this agent. However, no other MSSA strains containing this plasmid have been isolated so far in Brazil.

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# Risk Factors for Sustained Cholera Transmission, Juba County, South Sudan, 2014

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We conducted a case–control study to identify risk factors for the 2014 cholera outbreak in Juba County, South Sudan. Illness was associated with traveling or eating away from home; treating drinking water and receiving oral cholera vaccination were protective. Oral cholera vaccination should be used to complement cholera prevention efforts.

Cholera is an acute diarrheal disease caused by ingestion of food or water contaminated by the bacteria *Vibrio cholerae*, of which O1 is the most common serogroup in Africa (*1*,*2*). Although the proportion of global cholera cases reported from sub-Saharan Africa decreased from 93%–98% during 2001–2009 to 44% in 2013, cholera remains a major cause of disease epidemics in countries like South Sudan (*3*). In recent years, 4 major cholera outbreaks have occurred in South Sudan: in 2006, the number of cholera cases totaled 19,277 (case-fatality rate [CFR] 2.9%); in 2007, cases totaled 22,412 (CFR 1.8%); in 2008, cases totaled 27,017 (CFR 0.57%); and in 2009, cases totaled 48,035 (CFR 0.13%) (*4*–*6*; Ministry of Health, South Sudan, unpub. data). A previous case–control study conducted during the 2007 cholera outbreak in Juba County, South Sudan, showed that cholera was associated with being a visitor to Juba, having a water source close to the residence, and treating drinking water (because of inadequate treatment techniques); eating hot food was the only factor significantly associated with a lower risk of cholera (*7*).

On May 6, 2015, a cholera outbreak was confirmed in Juba County, South Sudan, during a major humanitarian crisis precipitated by political and ethnic tensions that deteriorated dramatically starting in December 2013. Epidemiologic investigations revealed that the outbreak started on April 23, 2014. We conducted a matched case–control study to identify risk factors for, and protective factors against, illness during the 2014 cholera outbreak in Juba County.

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## The Study

Cholera case-patients were identified from updated lists from Juba County's 5 cholera treatment centers (CTCs), 2 of which were located in camps for internally displaced persons (IDPs; i.e., persons who have left their homes but stayed within their country's borders). Preventive oral cholera vaccination was conducted in the 2 IDP camps before the outbreak began in Juba. A case-patient was defined as a Juba County resident ≥2 years of age who 1) had an acute illness characterized by ≥3 loose, watery stools within 24 hours or 2) was confirmed to be positive for *V. cholerae* infection by rapid diagnostic testing or culture during the cholera outbreak that began in Juba County on April 23, 2014.

For cholera case-patients enrolled in the study, a control matched by neighborhood, sex, and age was identified and invited to participate in the study. The study team traveled to the case-patient's village and worked with the local village leader or a social mobilization volunteer to identify a matching control from a household within a 100-m radius of the case-patient. A control was a Juba County resident ≥2 years of age with no history of clinical illness or no laboratory evidence of *V. cholerae* infection during this cholera outbreak. To match case-patients and controls by age, age groups of 3–5 years (e.g., 2–4, 5–9, and 65–69 years of age) were used.

A team of 19 trained research assistants administered a pretested, semistructured questionnaire and conducted environmental assessments to evaluate the use of safe drinking water, improved sanitation facilities, personal and food hygiene, and oral cholera vaccination. Using Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA), we calculated matched unadjusted and adjusted odds ratios by using bivariate and multivariate models, respectively, to identify risk factors for cholera. The study was approved by the Ministry of Health's ethics committee.

A total of 134 matched pairs of case-patients and controls were enrolled in the study during June 26–July 29 in 2014 (Figure). Of the 134 case-patients enrolled, 9 were confirmed by culture and 104 by a cholera rapid diagnostic test (OnSite Rapid Test; CTK Biotech, San

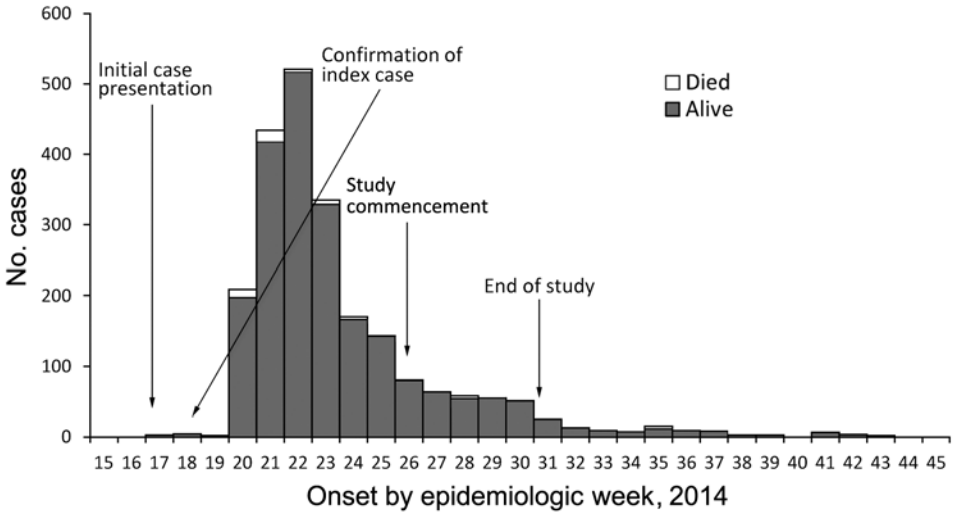


**Figure.** Timeline showing number of cholera cases (total cases = 2,260), deaths from cholera (total deaths = 43; case fatality rate 2.0%), and dates of study for cholera outbreak in Juba County, South Sudan, epidemiologic weeks 17–43 (April 23–October 20), 2014.

Diego, CA, USA); the remaining 21 were identified by epidemiologic linkage (i.e., a resident of Juba ≥2 years of age with ≥3 loose stools in 24 hours after the beginning of the cholera outbreak). Eleven cholera case-patients with no matching controls were excluded. Mean delay between admission to the CTC and interview after enrollment in the study was 21 days (range 0–55 days); most case-patients who were interviewed were admitted during the peak transmission phase of the outbreak (Figure). The distribution of age, sex, residence of origin, education level, and occupation were comparable among case-patients and controls (Table 1). Most (118 [88%]) case-patients enrolled in the study visited the CTC within 1 day of onset of cholera symptoms. All 134 patients had diarrhea; 112 (84%) had vomiting; 45 (34%) had abdominal cramps; 37 (28%) had some dehydration (i.e., any 2 signs of dehydration, including 1 major sign); and 31 (23%) had severe dehydration (23%).

Bivariate and multivariate analyses showed that persons who ate food outside their home before illness onset and those who traveled outside their home village (even within the county) before illness onset were significantly more likely to develop cholera (Table 2). Conversely, treating drinking water at home and receiving ≥2 doses of oral cholera vaccine (self-reported) were protective against cholera (Table 2). Eating outside the home as a risk factor in this cholera outbreak is consistent with findings from cholera outbreaks in Uganda and Haiti (*8,9*). Popular eating places in Juba County included roadside food vendors and restaurants in markets that did not meet minimum food hygiene standards yet remained open during the outbreak because public health inspection of eating establishments and a ban on roadside food vending were not uniformly enforced. Our study identified recent travel to cholera outbreak areas as a risk factor, also a finding consistently associated with cholera spread to new locations during previous cholera outbreaks (*10*).

Also, as reported in previous cholera outbreaks in South Sudan, Uganda, Haiti, and Zimbabwe, household chlorination of drinking water was associated with significantly lower risk for developing cholera in our study (*7,8,9,11*). In our study, water samples from case-patient households that did not chlorinate their drinking water showed evidence of contamination with fecal coliforms (>10 counts/100 mL). Similarly, water samples from water storage vessels in 2 case-patient households that did not

**Table 1.** Characteristics of cholera case-patients and controls during outbreak in Juba County, South Sudan, 2014

| Characteristic | Case-patients, no. (%), N = 134 | Controls, no. (%), N = 134 | p value |
|---|---|---|---|
| Age group, y | | | 0.99 |
| 0–9 | 59 (45) | 60 (45.5) | |
| 10–19 | 4 (3) | 5 (3.8) | |
| 20–29 | 28 (21.2) | 27 (20.6) | |
| 30–39 | 20 (15.2) | 21 (16) | |
| 40–49 | 14 (10.6) | 14 (10.7) | |
| 50–59 | 5 (3.8) | 4 (3.1) | |
| ≥60 | 1 (0.8) | 1 (0.8) | |
| Sex | | | 1.0 |
| M | 67 (50) | 67 (50) | |
| F | 67 (50) | 67 (50) | |
| Payam of origin* | | | 0.99 |
| Juba | 47 (35.0) | 47 (35.0) | |
| Northern Bari | 36 (27.0) | 36 (27.0) | |
| Rejaf | 30 (22.4) | 30 (22.4) | |
| Munuki | 6 (4.5) | 6 (4.5) | |
| Kator | 7 (5.2) | 7 (5.2) | |
| Others | 8 (5.9) | 8 (5.9) | |
| Education level | | | 0.85 |
| None | 87 (65.4) | 88 (65.7) | |
| Primary/tertiary | 46 (34.6) | 46 (34.3) | |
| Employment status | | | 0.18 |
| Unemployed | 100 (81.3) | 112 (86.2) | |
| Employed | 23 (18.7) | 18 (13.8) | |

*Payams are administrative divisions (counties) in South Sudan.

**Table 2.** Factors examined by using bivariate and multivariate analyses during cholera outbreak in Juba County, South Sudan, 2014*

| Factor | Case-patients, no. (%), N = 134 | Controls, no. (%), N = 134 | Unadjusted matched OR (95% CI) | Unadjusted matched p value | Adjusted matched OR (95% CI) | Adjusted matched p value |
|---|---|---|---|---|---|---|
| Ate outside home before illness | | | | | | |
| Yes | 42 (31.6) | 17 (14.0) | 6.5 (2.27–18.62) | <0.001 | 9.17 (1.89–44.41) | 0.006 |
| No | 91 (68.4) | 104 (86.0) | | | | |
| Traveled outside home village before onset of illness† | | | | | | |
| Yes | 37 (28.5) | 10 (7.9) | 13 (3.09–54.77) | <0.0001 | 10.14 (1.75–58.87) | 0.01 |
| No | 93 (71.5) | 117 (92.1) | | | | |
| Treated drinking water at home | | | | | | |
| Yes | 51 (38.3) | 58 (44.3) | 0.11 (0.02–0.55) | 0.04 | 0.10 (0.02–0.72) | 0.02 |
| No | 82 (61.7) | 73 (55.7) | | | | |
| Had 2 oral cholera vaccine doses‡ | | | | | | |
| Yes | 55 (41.7) | 78 (59.5) | 0.08 (0.02–0.35) | <0.001 | 0.10 (0.02–0.65) | 0.016 |
| No | 77 (58.3) | 53 (40.5) | | | | |

*The first 2 factors increased risk for cholera, whereas the other 2 factors decreased risk. OR, odds ratio.
†Travel from home to any area affected by cholera during the 2014 outbreak in South Sudan.
‡Self-reported.

treat their drinking water were contaminated with fecal coliforms (>10 counts/100 mL).

Oral cholera vaccination is known to confer protection from cholera (*12*). We found that oral cholera vaccination was associated with a significantly reduced risk of cholera infection and a vaccine effectiveness of 90% (Table 2).

Our findings are subject to several limitations that could potentially have confounded our results. These limitations include underreporting of high-risk behaviors, recall bias, potential misclassification of asymptomatic case-patients, narrow age ranges that caused difficulty in identifying matching controls, shared environmental risk factors (e.g., shared water source) for case-patients and controls, unmeasured variables (i.e., factors not measured in this study, such as being an IDP), and loss of oral cholera vaccination cards (i.e., vaccinations were self reported).

## Conclusions

For this cholera outbreak in South Sudan, we found that travel and eating outside the home were risk factors for becoming ill and that treating drinking water at home and getting oral cholera vaccination provided protection against illness. For cholera prevention and control in humanitarian crises, we recommend that global oral cholera vaccine stockpiles be enhanced so that preventive oral cholera vaccination can be used to augment traditional interventions, such as improved access to safe drinking water and public education about risk factors.

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# Detection of Mixed Infections with *Plasmodium* spp. by PCR, India, 2014

**Sri Krishna, Praveen K. Bharti, Himashu S. Chandel, Amreen Ahmad, Rajesh Kumar, Puspendra P. Singh, Mrigendra P. Singh, Neeru Singh**

In 8 malaria-endemic states in India, mixed *Plasmodium* spp. infections were detected by PCR in 17.4% (265/1,521) of blood samples that microscopy had shown to contain only *P. falciparum*. The quality of microscopy must be improved because use of PCR for detection of malaria parasites is limited in rural areas.

Five *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*) cause human malaria. Malaria is not uniformly distributed in India; 8 of the 35 states and union territories contain most malaria cases (*1*). Infections with *P. falciparum* and *P. vivax* occur at approximately equal frequencies (*2*–*4*). This finding increases the possibility of mixed infections, as reported in other countries, such as Peru (*5*), Papua New Guinea (*6*), Brazil (*7*), and Ethiopia (*8*).

In India, malaria control usually involves vector control with indoor residual spraying of insecticides and insecticide-treated bed nets, and chemotherapy with artemisinin-based combination therapy. Malaria diagnosis is based mainly on microscopic detection of parasites in peripheral blood smears from symptomatic persons. In addition, bivalent, rapid diagnostic tests (RDTs) are useful detection tools (*9*) but cannot differentiate *P. falciparum* moninfections from co-infections with other *Plasmodium* species (*2,3*). Moreover, genetic polymorphisms in diagnostic antigens limits detection by monoclonal antibodies. Misdiagnoses might also arise from gene deletions that prevent expression of proteins by the parasite (*10*). We report that a high proportion of mixed infections with 4 *Plasmodium* species detected by PCR in 8 states of India to which malaria is highly endemic were not detected by bivalent RDTs and microscopy.

## The Study

This study was approved by the Institutional review board of the National Institute for Research in Tribal Health (Jabalpur, India). Written informed consent was obtained from all participants or parents of children, according to Indian Council of Medical Research guidelines.

The study was conducted in 2 community health centers (CHCs), 1 in an area that had a high level of malaria endemicity and 1 that had a low level of malaria endemicity, in each of 8 states in India: Orissa, Chhattisgarh, Jharkhand, Maharashtra, Madhya Pradesh, Tripura, Gujarat, and Rajasthan (Figure 1; Table 1). Selected CHCs were located in different regions, and forest areas in these regions ranged from 13% in Jhabua (Madhya Pradesh) to 81% in Tripura. Elevation above sea level ranged from 13 m in Valsad (Gujarat) to 870 m in Koraput (Orissa). Inhabitants of most study areas were ethnic tribes (39%–87%). All areas had received 2 rounds of indoor residual spray (DDT/synthetic pyrethroid) as a vector control measure.

Blood samples were collected from persons with suspected malaria during July–December 2014 at malaria clinics in CHC hospitals at 15 sites. Microscopy and RDTs (Bioline Ag Malaria Pf/Pv Test; Standard Diagnostics Inc., Gyeonggi-do, South Korea) were performed at outpatient department clinics of CHCs, and molecular diagnosis (PCR and sequencing) was performed at the molecular parasitology laboratory at the National Institute for Research in Tribal Health.

For microscopy, thick and thin blood smears were prepared from finger prick blood samples, which were air-dried, fixed in methanol, and stained with Giemsa. A total of 100 thick blood smear fields were examined by using an oil immersion lens at 100× magnification before a sample was considered negative. Malaria parasite density was determined from thick blood smears by counting the number of parasites against 200 leukocytes (*11*). Microscopy was also performed on samples that had negative results by RDT. Blood smears were cross-checked by a senior laboratory technician. RDT was performed according to manufacturer's instructions (*9*) and was repeated for samples in which discordant results were obtained (e.g., microscopy positive, RDT negative).

Genomic DNA was isolated from samples that microscopy showed to contain only *P. falciparum* by using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). Species-specific nested PCRs that targeted the 18S rRNA gene were used to detect 4 malaria parasite species (*P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*) (*12*). *P. knowlesi* was detected by using a set of primers specific for the 18S rRNA gene (*13*), and differentiation of 2 subspecies of *P. ovale* (*P. curtisi* and *P. wallikeri*) was performed as described (*4*). PCR primers and conditions are shown in

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**Figure 1.** Fifteen community health centers in 8 states in India to which malaria is endemic. 1, Udaipur; 2, Dahod; 3, Valsad; 4, Jhabua; 5, Annupur; 6, Gondia; 7, Gadchiroli; 8, Jagdalpur; 9, Baikunthpur; 10, Koraput; 11, Rayagada; 12, Jaldega; 13, Bano; 14, Manu Bazar; 15, Shantir Bazar.

Table 2. An independent research assistant, who was unaware of microscopy or RDTs results, performed PCR on coded samples.

Of 1,521 samples determined by microscopy to be *P. falciparum* moninfections, PCR confirmed results for 1,256 (83%). However, PCR showed mixed infections with *P. falciparum* and *P. vivax* in 239 (16%) samples; *P. falciparum* and *P. malariae* in 19 (1%) samples; *P. falciparum* and *P. ovale* in 6 (0.4%) samples; and *P. falciparum*, *P. malariae*, and *P. ovale* in 1 (0.1%) sample (Table 1). Microscopy could not identify these mixed infections (17.4% [265/1,521]). PCR amplification of DNA from 4 *Plasmodium* species is shown in Figure 2.

Secondary microscopic analysis of blood smears by a second technician showed that only 22/239 (9.2%) samples contained mixed infections with *P. falciparum* and *P. vivax*. PCR analysis showed that the highest prevalence of mixed infections with *P. falciparum* and *P. vivax* was in Jharkhand (25.5%, 55/216), followed by Madhya Pradesh (20.8%, 47/226), Rajasthan (18.6%, 26/140), Orissa (15%, 40/267), and Tripura (15%, 19/127), and Chhattisgarh (10.7%, 23/214). The lowest prevalences were in Maharashtra (9%, 21/234) and Gujarat (8.2%, 8/97).

Mixed infections with *P. falciparum* and *P. malariae* were found in all 8 states, although in small numbers. Mixed infections with *P. falciparum* and *P. ovale* were found in only 4 states, particularly at CHCs in areas to which malaria was highly endemic. Of 7 mixed infections that contained *P. ovale*, 5 contained *P. ovale curtisi* and 2 contained *P. ovale wallikeri*. *P. knowlesi* was not found in any state.

## Conclusions

This study was conducted 8 states in India that contain 80% of malaria cases (85% of which are caused by *P. falciparum*) and 70% of deaths caused by malaria in the entire country (*1*). Misdiagnosis by microscopy occurs because in mixed infections there is a tendency of 1 parasite to predominate and microscopy usually does not detect low numbers of other parasites (*6*). Thus, rare malaria parasites and mixed infections are underestimated through routine

**Table 1.** Characteristics of mixed infections with 4 *Plasmodium* species identified by PCR for 1,521 blood samples that were *P. falciparum*–positive by microscopy in 8 malaria-endemic states, by district, India, 2014*

| District (state) or variable | CHC | Period | No. infections | | | | | Odds ratio (95% CI), p value† | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *Pf* | *Pf* + *Pv* | *Pf* + *Pm* | *Pf* + *Po* | *Pf* + *Pm* + *Po* | Mixed (*Pf* + *Pv*) | Mixed (all pooled) |
| Koraput (OD) | Bandhugaon | Jul–Aug | 188 | 35 | 4 | 2 | 0 | – | – |
| Rayagada (OD) | Jagannathpur | Jul–Aug | 31 | 5 | 2 | 0 | 0 | 1.8 (1.0–3.1), 0.004 | 2.0 (1.2–3.4), 0.0091 |
| Simdega (JH) | Jaldega | Aug–Nov | 82 | 41 | 0 | 2 | 0 | – | – |
| Simdega (JH) | Bano | Aug–Nov | 76 | 14 | 0 | 0 | 1 | 3.5 (2.0–6.0), <0.0001 | 3.4 (2.0–5.8), <0.0001 |
| Jagdalpur (CG) | Maharani Hospital | Jul–Oct | 178 | 23 | 2 | 1 | 0 | – | – |
| Baikunthpur (CG) | District Hospital | Jul–Nov | 10 | 0 | 0 | 0 | 0 | 1.2 (0.7–2.3), 0.5292 | 1.3 (0.7–2.3), 0.4324 |
| Jhabua (MP) | Ranapur | Sep–Oct | 92 | 29 | 3 | 1 | 0 | – | – |
| Anuppur (MP) | Pushprajgarh | Sep–Nov | 82 | 18 | 1 | 0 | 0 | 2.7 (1.5–4.7), 0.0004 | 2.7 (1.6–4.7), 0.0001 |
| Gadchiroli (MH) | Malewada | Sep–Nov | 108 | 6 | 0 | 0 | 0 | – | – |
| Gondia (MH) | Darekasa | Sep–Nov | 103 | 15 | 2 | 0 | 0 | 1 (Ref) | 1 (Ref) |
| Udaipur (RJ) | Bekaria | Sep–Dec | 112 | 26 | 2 | 0 | 0 | 2.3 (1.2–4.3), 0.0068 | 2.3 (1.3–4.2), 0.0056 |
| Dahod (GJ) | Devgadh Baria | Sep–Dec | 77 | 8 | 2 | 0 | 0 | – | – |
| Valsad (GJ) | Lavkar | Sep–Nov | 10 | 0 | 0 | 0 | 0 | 0.9 (0.4–2.1), 0.8316 | 1.1 (0.5–2.3), 0.8946 |
| South Tripura (TR) | Manubazar | Oct–Dec | 41 | 4 | 0 | 0 | 0 | – | – |
| South Tripura (TR) | Santirbazar | Oct–Dec | 66 | 15 | 1 | 0 | 0 | 1.8 (0.9–3.5), 0.084 | 1.7 (0.9–3.3), 0.0978 |
| Total | NA | NA | 1,256 | 239 | 19 | 6 | 1 | NA | NA |
| Median no. parasites/μL | NA | NA | 1,897.3 | 1,600 | 1,273.6 | 1,180 | 4,040 | NA | NA |
| Range | NA | NA | 35–1,785,714 | 40–380,464 | 31–56,818 | 200–124,118 | NA | NA | NA |
| p value | NA | NA | Ref | 0.138 | 0.251 | 0.439 | NA | NA | NA |

*CHC, community health center; *Pf*, *P. falciparum*; *Pv*, *P. vivax*; *Pm*, *P. malariae*; *Po*, *P. ovale*; OD, Odisha; JH, Jharkhand; CG, Chhattisgarh; MP, Madhya Pradesh; MH, Maharashtra; Ref, reference; RJ, Rajasthan; GJ, Gujrat; TR, Tripura; NA, not applicable.
†–, indicates that analysis was conducted after data for the 2 districts in that state were combined.

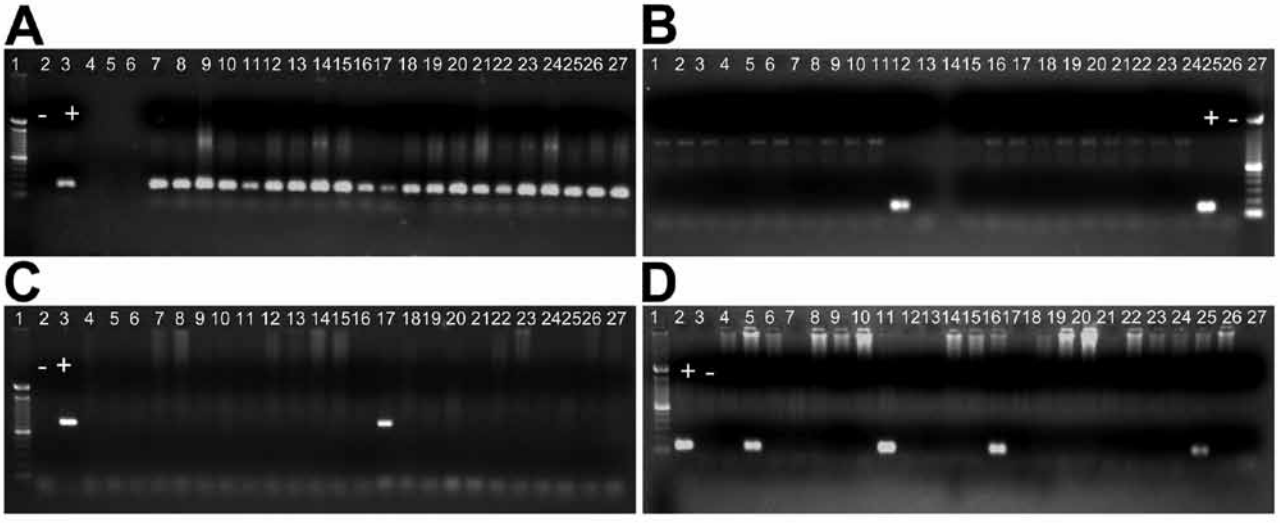


**Figure 2.** Identification of *Plasmodium* spp. by nested PCR at 15 community health centers in 8 states in India to which malaria is endemic. A) *Plasmodium falciparum* (205-bp fragment). Lane 1, molecular mass marker; lane 2, negative (–) control; lane 3, positive (+) control; lanes 7–27, positive samples; lanes 5 and 6, negative samples. B) *P. malariae* (144-bp fragment). Lane 25, + control; lane 26, – control; lane 27, molecular mass marker; lane 12, positive sample; lanes 1–11, 13–24, negative samples. C) *P. ovale* (800-bp fragment). Lane 1, molecular mass marker; lane 2, – control; lane 3, + control; lane 17, positive sample; lanes 4–16, 18–27, negative samples. D) *P. vivax* (120-bp fragment). Lane 1, molecular mass marker; lane 2, + control; lane 3, – control; lanes 5, 11, 16, and 25, positive samples; lanes 4, 6–10, 12–15, 17–24, 26, and 27, negative samples.

**Table 2.** Characteristics of PCR primers specific for 18S rRNA gene of *Plasmodium* spp., India*

| Genus or species | Primer | Sequence, 5′→3′ | PCR product, bp | PCR Program | | | | | | No. cycles |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Denaturation | | Annealing | | Elongation | | |
| | | | | Temp, °C | Time, min | Temp, °C | Time, min | Temp ,°C | Time, min | |
| *Plasmodium* | F | TTAAAATTGTTGCAGTTAAAACG | 1,200 | 94 | 1 | 58 | 2 | 72 | 2 | 25 |
| | R | CCTGTTGTTGCCTTAAACTTC | 1,200 | 94 | 1 | 58 | 2 | 72 | 2 | 25 |
| *P. falciparum* | F | TTAAACTGGTTTGGGAAAACCAAATATATT | 205 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| | R | ACACAATGAACTCAATCATGACTACCCGTC | 205 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| *P. vivax* | F | CGCTTCTAGCTTAATCCACATAACTGATAC | 120 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| | R | ACTTCCAAGCCGAAGCAAAGAAAG TCCTTA | 120 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| *P. malariae* | F | ATAACATAGTTGTACGTTAAGAATAACCGC | 144 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| | R | AAAATTCCCATGCATAAAAAATTATACAAA | 144 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| *P. ovale* | F | ATCTCTTTTGCTATTTTTTAGTATTGGAGA | 800 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| | R | GGAAAAGGACACATTAATTGTATCCTAGTG | 800 | 94 | 1 | 58 | 2 | 72 | 2 | 30 |
| *P. knowlesi* | F | CAGAGATCCGTTCTCATGATTTCCATGG | 209 | 95 | 0.5 | 57 | 0.5 | 72 | 0.75 | 35 |
| | R | CTRAACACCTCATGTCGTGGTAG | 209 | 95 | 0.5 | 57 | 0.5 | 72 | 0.75 | 35 |
| *P. ovale* | F | CTACTTGACATTTCTACTTACA | 938 | 95 | 1 | 50 | 1 | 72 | 1 | 35 |
| | R | CGTTCTTGATTAATGGAAGTAT | 938 | 95 | 1 | 50 | 1 | 72 | 1 | 35 |
| *P. ovale* (*curtisi* and *wallikeri*) | F | GCTGTAGCTAATACTTGCTTTA | 827 | 95 | 1 | 55 | 1 | 72 | 1 | 25 |
| | R | TTCACCTCTGACATCTGAATC | 827 | 95 | 1 | 55 | 1 | 72 | 1 | 25 |

*F, forward; R, reverse.

microscopy and RDTs (*2*–*6*), and misidentification of malaria parasites could prolong parasite clearance time and lead to anemia and drug resistance (*14*). A high proportion of mixed infections with *P. vivax* and *P. falciparum* have been reported in India (*15*). However, in that study, Gupta et al. did not look for *P. malariae* or *P. ovale* and their sample size was small (180 persons).

Our study had some limitations. Monoinfections or mixed infections were not verified by PCR if parasitemia levels were too low to be detected by microscopy or RDTs. Thus, mixed infections with parasitemia levels below the limit of detection of microscopy or RDTs would not have been detected. Detailed studies in different ecosystems during different transmission seasons and large sample sizes are required for a more accurate picture of mixed infections with common and uncommon parasite species, clinical epidemiology, adverse effects, relapse, and recrudescence.

Our results highlight the role of mixed infections, particularly those with *P. vivax*, *P. malariae*, and *P. ovale*, which are not detected accurately by microscopy or RDTs. Although *P. vivax* and *P. ovale* are responsible for relapses (*4*), *P. malariae* is sustained at low rates among sparse and mobile human populations for decades, thus facilitating transmission by mosquitoes (*2*). Our results also emphasize a major concern in the diagnosis of malaria by microscopy or RDTs and has serious repercussions for malaria epidemiology and subsequent control. These findings indicate the need to improve quality of microscopy and RDTs because PCR techniques are expensive. Until PCR becomes much less expensive and more available as a point-of-care test for field testing, its use will be limited for malaria detection.

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# Acute Flaccid Paralysis Associated with Novel Enterovirus C105

**Liana M. Horner, Melinda D. Poulter, J. Nicholas Brenton, Ronald B. Turner**

An outbreak of acute flaccid paralysis among children in the United States during summer 2014 was tentatively associated with enterovirus D68 infection. This syndrome in a child in fall 2014 was associated with enterovirus C105 infection. The presence of this virus strain in North America may pose a diagnostic challenge.

During the summer of 2014, the Centers for Disease Control and Prevention (CDC) reported an unusual increase in the frequency of acute flaccid myelitis among children in the United States (*1*). A case definition was developed, and clinicians were urged to report new cases to CDC and state health departments. This outbreak occurred coincidentally with an outbreak of respiratory disease caused by enterovirus D68 (EV-D68) (*2*). The simultaneous occurrence of the neurologic disease and the widespread occurrence of an unusual respiratory enterovirus syndrome raised suspicion that the 2 outbreaks might be linked (*3*,*4*). We report a case that met the CDC case definition of acute flaccid myelitis but was associated with isolation of a novel enterovirus, C105, which has been previously isolated from a patient with flaccid paralysis. The presence of this virus strain in North America may contribute to the incidence of flaccid paralysis and may also pose a diagnostic challenge in clinical laboratories.

## The Case

The patient was a 6-year-old previously healthy girl examined at the University of Virginia Children's Hospital in October 2014 for acute onset of progressive right upper extremity weakness. Within the 2 weeks before the patient's presentation to the hospital, she and her family members had been ill with a mild cough and rhinorrhea; 4 days before presentation, the patient had experienced low-grade fever (100.4°F), frontal headache, fatigue, and intermittent pain in the right ear and right axilla. The fever lasted only 1 day; the cough, fatigue, and headache improved over the next 2 days, but the patient continued to report right arm pain. On the day before seeking care, her parents observed that she had a right shoulder droop and difficulty using her right hand. No associated visual or mental status changes; difficulty with speech, swallowing, or respiration; or bowel/bladder disturbance were noted. Physical examination detected right upper extremity weakness; absent right biceps, triceps, and brachioradialis deep tendon reflexes; and a diminished right patellar reflex. Muscle strength was more severely affected in the proximal than in the distal right upper extremity. Sensation was intact. A diffuse papular rash was noted on the patient's back.

Magnetic resonance (MR) images of the spine revealed longitudinally extensive gray matter hyperintensity within the central cord at C3–7 and T11–12/L1 with associated edema (Figure). An MR image of the brain was unremarkable. Examination of cerebrospinal fluid (CSF) detected $5 \times 10^6$ leukocytes/L (82% lymphocytes), $0.9 \times 10^6$ erythrocytes/L, 3 mmol glucose/L, and 280 mg protein/L. The IgG index R-value (relative IgG/albumin ratio in CSF and serum) was mildly elevated at 0.73, but oligoclonal bands were absent. Testing of a nasopharyngeal swab specimen with the xTAG Respiratory Viral Panel (Luminex, Austin, TX, USA) produced positive results for picornavirus. PCR for enterovirus in CSF, performed by using the XpertEV test (Cepheid, Sunnyvale, CA, USA) was negative. Subsequent confirmatory testing at the Division of Consolidated Laboratories for the Commonwealth of Virginia also produced negative results. Subsequent testing of a nasopharyngeal swab specimen by sequence analysis of the viral protein 1 capsid gene revealed the presence of enterovirus C105 (*5*). Culture of a fecal specimen produced negative results for enterovirus. Other negative results were obtained from serologic testing for *Borrelia burgdorferi*, arboviruses, and neuromyelitis optica IgG.

The patient received intravenous immunoglobulin, 2 g/kg divided daily over 5 days, but did not respond. She continued to report intermittent pain in her right arm, low-back pain, and bilateral thigh pain when walking. During her hospital stay, the patient's weakness remained stable (no substantial progression or improvement). After discharge, her pain spontaneously resolved, and 8 months after illness onset (most recent follow-up visit), her proximal right arm weakness improved and the strength in her distal right arm had almost resolved.

## Conclusions

Enterovirus C105 was first detected in 2010 in patients from Peru and the Republic of Congo; subsequent reports suggest that the virus is circulating worldwide (*6*–*9*). The enterovirus C species includes the polioviruses and 11

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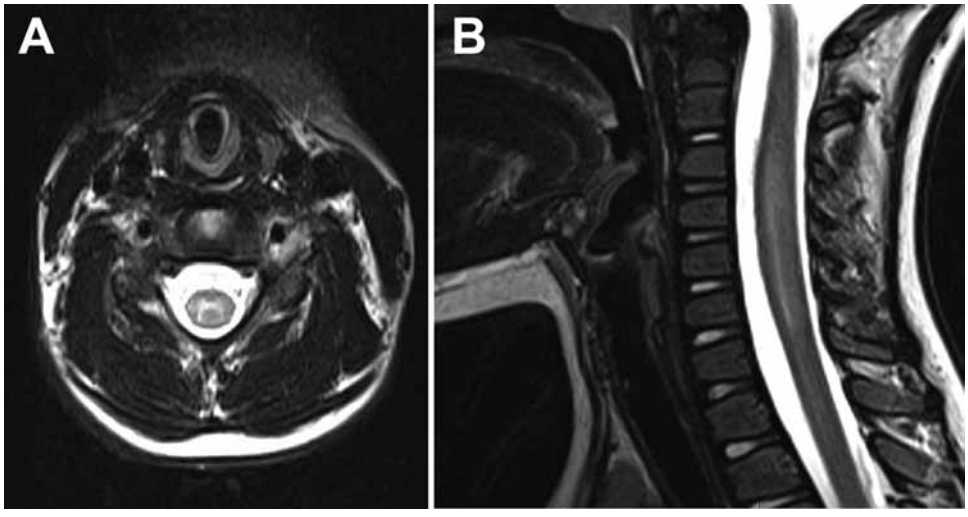


**Figure.** Magnetic resonance imaging of 6-year-old girl with flaccid paralysis and enterovirus C105 infection, Virginia, USA, October 2014. A) Axial T2-weighted image of the cervical spine demonstrating abnormal hyperintensity of the central gray matter (right to left). B) Sagittal T2-weighted image of the cervical spinal cord demonstrating faint longitudinally extensive central hyperintensity and associated cord edema.

enterovirus serotypes previously classified as coxsackie A viruses. Enterovirus C105 seems to be a member of a recently detected subspecies that is distinguished from the other enterovirus C viruses by a divergent 5′-untranslated region (UTR) (*10*). The clinical spectrum associated with these recent isolates is still poorly defined. Most isolates have been associated with respiratory syndromes, but the isolate from the Republic of Congo was associated with fatal acute flaccid paralysis.

Detecting the virus in clinical specimens may be challenging because of the divergence of the 5′-UTR in members of this newly emerging subspecies of enterovirus C (*10*,*11*). Many broad-specificity enterovirus real-time reverse transcription PCRs target conserved regions of the 5′-UTR. The sequence divergence in the subspecies occurs in regions that are targeted by these diagnostic assays and may interfere with recognition of the virus by the primers. For the patient we report, virus in the nasopharyngeal swab sample was identified as a picornavirus by the xTAG Respiratory Virus Panel and by sequence analysis of the viral protein 1 capsid gene (*5*).

In the United States, the outbreak of acute flaccid myelitis that began in the summer of 2014 affected 118 children (http://www.cdc.gov/ncird/investigation/viral/sep2014/investigation.html). The case definition for the outbreak is acute onset of focal limb weakness associated with a spinal cord lesion restricted to the gray matter (according to MR images) in a child <21 years of age. The patient reported here met this case definition. A detailed report of 88 of these children noted that most (81%) had experienced a preceding respiratory illness, similar to that described by the patient reported here (*12*). Despite the suspicions that EV-D68 may be a cause of the neurologic syndrome (*3*,*4*), early reports indicate that EV-D68 was detected in only 8 (20%) of 41 of the flaccid myelitis patients tested, and no enterovirus has been detected in spinal fluid (*12*). EV-D68 was not detected in specimens from the patient reported here, and EV-D68 was not epidemic in central Virginia. However, we did not have access to convalescent serum samples for antibody testing, and it is not possible to definitively exclude the possibility of an undetected co-infection.

EV-D68 is unusual in that, although it is a member of the enterovirus D species, this virus has phenotypic characteristics that are more consistent with rhinoviruses. The virus is acid labile and preferentially grows at 33°C, characteristics that have been used to classify picornaviruses as rhinoviruses (*13*). The virus also seems to be similar to rhinoviruses in its propensity to cause an afebrile respiratory syndrome and to exacerbate asthma symptoms in asthma patients (*2*). Rhinoviruses are not associated with systemic disease, and virus replication is limited to the respiratory tract, a characteristic that has been attributed to the temperature sensitivity of the virus. Although cases of flaccid paralysis associated with isolation of EV-D68 from spinal fluid have been reported, the role of EV-D68 in the current outbreak remains to be determined. As the results from this case indicate, it is possible that other viral pathogens with neurovirulence may be contributing to the outbreak.

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# Transmission Risk from Imported *Plasmodium vivax* Malaria in the China–Myanmar Border Region

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Malaria importation and local vector susceptibility to imported *Plasmodium vivax* infection are a continuing risk along the China–Myanmar border. Malaria transmission has been prevented in 3 border villages in Tengchong County, Yunnan Province, China, by use of active fever surveillance, integrated vector control measures, and intensified surveillance and response.

A sharp increase in imported malaria cases has made preventing reintroduction of malaria in China a major challenge (*1*). High importation risk from Myanmar, where malaria is endemic, and wide distribution and abundance of malaria vectors in the China–Myanmar border region sustain risk for secondary infections among local populations. Tengchong County in Yunnan Province, bordering Myanmar, reported the highest number of imported malaria cases during 2010–2014 in China. A recent field survey indicated secondary transmission from imported *Plasmodium vivax* malaria in this region (*2*). To inform malaria elimination efforts in the region, we assessed local vectorial capacity and evaluated risk for secondary infections arising from malaria importation.

## The Study

Three villages (Manduo, Luoping, and Tuofeng; Figure) in Tengchong County, located in the westernmost part of Yunnan Province, were selected for study because of their 2011–2013 malaria incidence, ecologic features related to malaria transmission (i.e., altitude and proportion of land used for rice cultivation), and housing and economic status. The villages range in altitude from 1,276 to 1,893 meters and have 1,115 households and a population of 4,904 (detailed methods in online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/10/15-0679-Techapp1.pdf).

During 2011–2013, a total of 24 *P. vivax* malaria cases were reported from the study villages to the Chinese Information System for Disease Control and Prevention; all were classified as imported (online Technical Appendix), as determined by patients' travel history (*3*). All patients were adult men 18–49 years of age, and most worked in business or mining. Patients were radically cured with a regimen of chloroquine and primaquine, as recommended by national treatment guidelines. Malaria vulnerability or importation risk (i.e., incidence of imported malaria cases per 1,000 population per year) averaged 1.6 cases in the study area during 2011–2013 (online Technical Appendix Table 1). The average interval between symptom development and diagnosis of malaria was 2.4 days; average interval between diagnosis and treatment was 1.5 days (Table 1). All cases were reported within 1 day and investigated within 3 days (Table 1).

Health workers performing active fever surveillance during the 2013 transmission season (May–September) visited each house at 2-week intervals, conducted a total of 7,680 household interviews and 38,960 interviews with village residents, and collected 399 blood samples from persons with history of fever during the previous 2 weeks. A total of 268 (67.2%) samples were from local residents who reported no travel outside Tengchong County within the past 2 weeks; 131 were from mobile populations reporting travel. *P. vivax* isolates were detected by microscopy and PCR in 10 (7.6%) persons in the mobile population; no malaria infection was found in local persons (online Technical Appendix Table 2).

To estimate human biting rates, mosquitoes were collected in each study village every 2 weeks by using volunteer outdoor human-landing catches during May–September 2013 (online Technical Appendix). A total of 5,576 mosquitoes were caught; most (95%) were *Anopheles sinensis* mosquitoes. The average number of mosquitoes landing on a single person per night (human landing rate)

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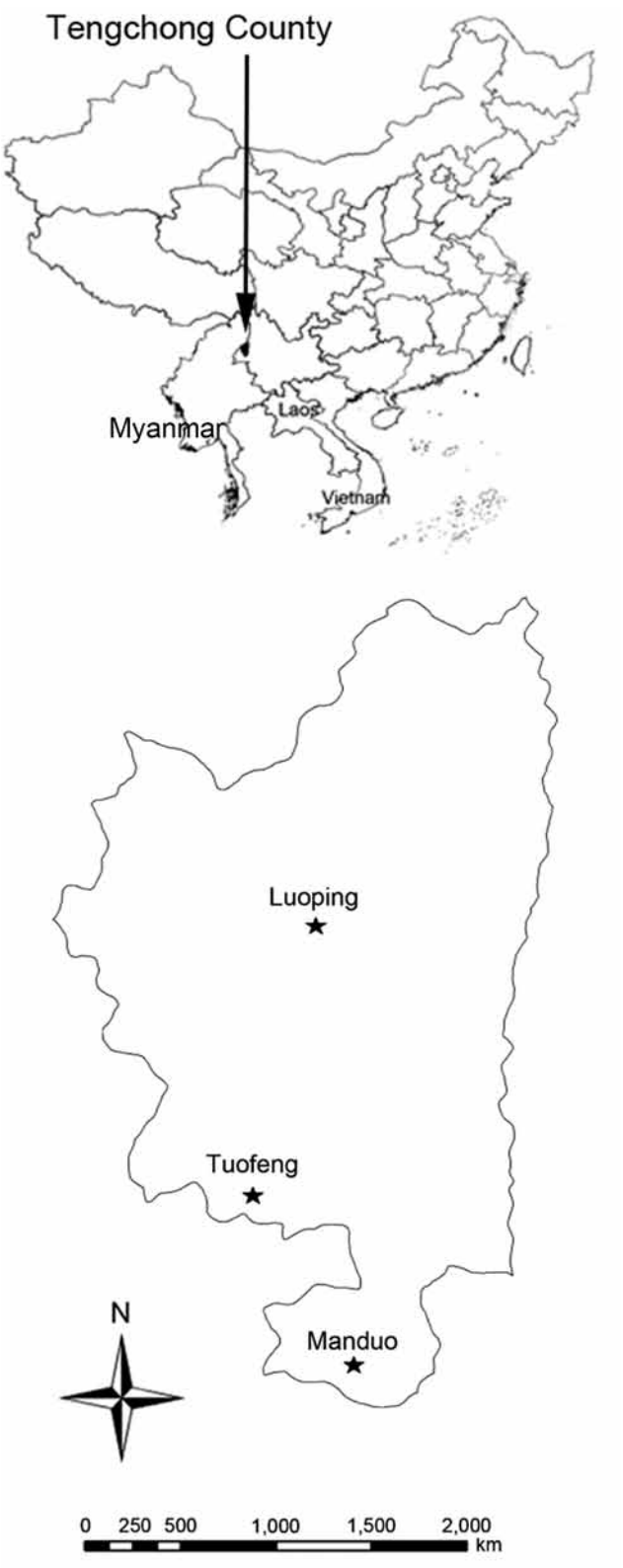


**Figure.** Location of 3 villages in Tengchong County, Yunnan Province, China, in which study of transmission risk from imported *Plasmodium vivax* malaria was conducted. Inset shows location of Tengchong County along the China–Myanmar border.

was 2.7 (Table 2; online Technical Appendix Table 3). The ratio of parous to nulliparous mosquitoes (multiparous ratio) of the *An. sinensis* mosquitoes tested for the villages of Manduo, Luoping, and Tuofeng was 0.52, 0.54, and 0.61, respectively (online Technical Appendix Table 4). The average human blood index (i.e., proportion of tested mosquitoes with ingested human blood) was 3.8% in the surveillance areas (Table 2; online Technical Appendix Table 5). Average vectorial capacity (i.e., expected number of new human infections from 1 infected person within 1 day, assuming all mosquitoes with sporozoites are potentially infective) of *An. sinensis* mosquitoes was 0.02 (Table 2), indicating that ≈50 cumulative days would be needed for transmission of malaria from an infected person to another person, assuming that all female mosquitoes biting malaria-infected persons become infected and transmit. The proportion of tested field-caught *An. sinensis* mosquitoes found to have ingested human blood (i.e., blood feeding rate) was 15.6% (31/199). The proportion of infected mosquitoes was 16.1% (5/31), the rate of susceptibility of local *An. sinensis* mosquitoes to imported *P. vivax* infection in this study.

## Conclusions

*An. sinensis* mosquitoes were the main local vector for *P. vivax* and were widely distributed throughout this study area. The vector's susceptibility to imported *P. vivax* infection indicates potential for *P. vivax* malaria to be sustained in this region. Although a recent field survey showed secondary transmission from patients with imported *P. vivax* malaria in other villages in Tengchong County (*2*), we identified no secondary infections in the study villages.

*An. sinensis* mosquitoes are the most widely distributed malaria vector in China but have relatively low susceptibility to parasites compared with other malaria vectors (*4*). We confirmed that *An. sinensis* mosquitoes were susceptible to imported *P. vivax* infection; however, vectorial capacity of *An. sinensis* mosquitoes was lower than it was during the 1990s (0.05) in the same region (*5*) and much lower than reported in central China (0.3) in the 1980s (*6*). The reduced vectorial capacity in the study region since the 1990s is likely attributable to the change in the dominant malaria vector species. Historically, *An. kunmingensis* mosquitoes were the main malaria vector in Tengchong County and accounted for 77% of total malaria vector density; its vectorial capacity (0.3) was ≈10 times higher than that of *An. sinensis* mosquitoes (0.03) in the 1980s (*7*). Few *An. kunmingensis* mosquitoes (<5% of total malaria vectors) were captured in our study, likely because of extensive residual insecticide use and improved housing, which reduce contact with this mosquito and vectorial capacity. High coverage (>90%) of long-lasting insecticidal nets and indoor residual spraying

**Table 1.** Malaria case management and response in 3 villages in the China–Myanmar border region, 2011–2013

| Village | No. cases | Days from illness onset to diagnosis | Days from diagnosis to treatment | Reported within 1 d, % | Investigated within 3 d, % | Febrile, screened within 7 d, no. | Additional cases identified, no. |
|---|---|---|---|---|---|---|---|
| Manduo | 8 | 3.2 | 1.6 | 100 | 100 | 124 | 0 |
| Luoping | 11 | 1.8 | 1.5 | 100 | 100 | 224 | 0 |
| Tuofeng | 5 | 2.2 | 1.5 | 100 | 100 | 99 | 0 |
| Total | 24 | 2.4 | 1.5 | 100 | 100 | 447 | 0 |

has been achieved in southern China along the Myanmar border since implementation of the National Malaria Elimination Program in July 2010.

In early 2012, a real-time surveillance system and response strategy ("1-3-7") was rolled out nationally (*8*) and substantially improved timeliness of malaria surveillance and response activities. During 2011–2013, all malaria cases in the study area were reported within 1 day and investigated within 3 days, and screening of persons with fever was conducted within 7 days. Other countries reporting few days (range 3.0–8.2 days) before testing and treatment of imported malaria reported similar results and no subsequent secondary infections (*9–11*). In our study, additional testing by PCR for those reporting fever found no subpatent infections (i.e., slight infections with low parasitemia), a finding consistent with a recent review that showed no difference between PCR and microscopy for detecting parasites in symptomatic persons. However, a large proportion of *P. vivax* infections were subpatent in a cross-sectional survey of the general population in China (*12*).

Rigorous evaluation of malaria elimination programs is essential for continuously improving the programs, targeting limited resources, and maintaining financial and political support. We used robust entomologic and epidemiologic metrics to assess malaria elimination in a border region. Many national malaria control programs lack capacity to conduct entomologic surveillance to assess vectorial capacity. Although our study shows evidence of successful malaria elimination, additional validated metrics to ascertain success are needed. Recent efforts to compare the basic reproductive rate (total number of malaria cases derived from 1 infective case and distributed by mosquitoes in the absence of immunity) for imported versus local malaria cases provide a more nuanced and stable metric for measuring malaria elimination (*13*). Because this region has a unique ecology and distinct mosquito species composition, our findings need further validation to determine whether they can be extrapolated to other areas of China's border region (*14*).


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**Table 2.** Vectorial capacity of *Anopheles sinensis* mosquitoes in 3 villages in the China–Myanmar border region, May–September 2013*

| Village | Human landing rate† | Human blood index‡ | Mosquito biting habits§ | Daily survival rate¶ | Days of sporogonic development# | $p^n$** | Survival, d†† | Receptivity‡‡ |
|---|---|---|---|---|---|---|---|---|
| Manduo | 1.2 | 0.04 | 0.02 | 0.8 | 12.4 | 0.1 | 5.4 | 0.01 |
| Luoping | 4.9 | 0.04 | 0.02 | 0.8 | 14.0 | 0.09 | 5.7 | 0.05 |
| Tuofeng | 2.0 | 0.03 | 0.01 | 0.9 | 23.3 | 0.04 | 7.2 | 0.01 |
| Total | 2.7 | 0.04 | 0.02 | 0.9 | 16.6 | 0.08 | 6.1 | 0.02 |

*Vectorial capacity, expected number of new human infections from 1 infected person within 1 day, assuming all mosquitoes with sporozoites are potentially infective. HBI, human blood index (proportion of tested mosquitoes having ingested human blood.
†Average number of mosquitoes landing on a single person per night (*ma*).
‡Proportion of tested mosquitoes having ingested human blood.
§Human blood index divided by days needed to complete gonotrophic cycle (cycle of taking a blood meal and laying eggs).
¶Probability (*p*) of a mosquito surviving 1 whole day.
#Time (*n*) needed for parasites to complete development from ingested gametocytes during blood meal to sporozoites in salivary glands, when parasites are transmissible to humans.
**Fraction of infected mosquitoes after duration of sporogony.
††Duration of vector's life, in days, after surviving the extrinsic incubation period, calculated as the negative logarithmic reciprocal of the daily survival rate: $1/-\ln(p)$.
‡‡Expressed by the vectorial capacity index: $ma^2(p^n/-\ln(p))$.

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# Local and International Implications of Schistosomiasis Acquired in Corsica, France

**Philippe Gautret, Frank P. Mockenhaupt, Frank von Sonnenburg, Camilla Rothe, Michael Libman, Kristina Van De Winkel, Emmanuel Bottieau, Martin P. Grobusch, Davidson H. Hamer, Douglas H. Esposito, Philippe Parola, Patricia Schlagenhauf, for the GeoSentinel Surveillance Network**[1]

We report 11 cases of schistosomiasis in international travelers who had bathed in rivers in Corsica, France, during 2012–2014. The infections were diagnosed in 2014 and reported to the GeoSentinel Surveillance Network and European Travel Medicine Network. Travelers can be sentinels for emerging infections; thus, this situation warrants a concerted human and veterinary epidemiologic response.

In 2014, reports were received of several cases of *Schistosoma haematobium* trematode infection acquired in Corsica, a Mediterranean French island. The first patient was a child from Germany who had traveled to southern Corsica in August 2013 and had no other known exposures. Medical examination showed that the child had gross hematuria; he received a diagnosis of urinary schistosomiasis (*1*). Serologic test results were positive for 4 of 5 asymptomatic family members who had also traveled to Corsica and bathed in the Cavu River, near Porto-Vecchio. Eleven additional cases of urinary schistosomiasis were reported among mainland French tourists who bathed in the Cavu River during August 2011–August 2013 (*2–4*). All cases were identified during the chronic phase of the disease.

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Following a national screening campaign in France, ≈100 additional schistosomiasis cases were described over 2 years among Corsica residents and visitors from other parts of France. In addition, a competent intermediate host snail, *Bulinus truncatus*, was identified in rivers in Corsica (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/10/15-0881-Techapp1.pdf). To elucidate the epidemiologic characteristics of schistosomiasis in Corsica, we determined the number of and clinical findings for urinary schistosomiasis cases among international travelers to Corsica.

## The Patients

To find cases, we searched the GeoSentinel Surveillance Network database (*5*) for urinary schistosomiasis diagnoses for international travelers with freshwater (river) exposure in Corsica during 1996 through March 2015. We also informally extended the search to members of the European Travel Medicine Network (EuroTravNet, http://www.istm.org/eurotravnet). GeoSentinel's data-collection protocol is for public health surveillance, so human subjects' review board clearance was not needed. According to local institutional review boards at sites in Berlin and Munich, Germany, our use of supplemental data not available in the GeoSentinel database conforms to the human subject protection guidelines at these sites. Individual patient consent was obtained at sites in Hamburg, Germany; Ghent, Belgium; and Montreal, Quebec, Canada.

We identified 11 records with diagnoses of schistosomiasis acquired in Corsica during 1996–March 2015 (Table). Patients resided in Germany, Belgium, or Canada and had traveled to Corsica during 2012–2014; some had also traveled to Corsica before 2012. No patients reported other exposure to freshwater in any other country where schistosomiasis is known to be endemic. All 11 patients reported bathing in rivers in Corsica: 7 persons (6 from Germany, 1 from Canada) bathed in the Cavu River; a familial cluster of 3 persons from Berlin bathed in the Gaglioli, Solenzara, and Restonica Rivers; and a Belgian tourist bathed in the Osu River.

All infections were in asymptomatic persons who sought screening in 2014 after seeing/hearing public health warnings regarding the risk for acquiring schistosomiasis after freshwater exposure in Corsica. Four cases were in children <15 years of age; 1 was in a 17-year-old girl. Two

[1]Additional members of the GeoSentinel Surveillance Network who also contributed data are listed at the end of this article.

**Table.** Characteristics for 11 international travelers with urinary schistosomiasis diagnosed in 2014 and acquired in Corsica, France*

| Patient age, y/sex | Reporting site | Travel to Corsica | | First clinic visit, 2014 | Eosinophils, cells/μL | Serologic test result | | Eggs in urine | Infection status† |
|---|---|---|---|---|---|---|---|---|---|
| | | 2014 | Before 2014 | | | First-line (antibody titer, IU) | Second-line | | |
| 7/M | Berlin, Germany | None | 2013 | Jun 5 | 1,415 | Pos (26.0)‡ | ELISA and IHA Neg§ | Neg | Probable |
| 52/M | Berlin | None | 2012 | Jul 18 | 192 | Borderline (11.0)‡ | ELISA and IHA Neg§ | Neg | Suspected |
| 29/F | Ghent, Belgium | None | 2013 | Aug 29 | 560 | Pos (1.1)¶ | IHA Neg# | Neg | Probable |
| 17/F | Hamburg, Germany | July–August | None | Sep 8 | 45 | Weak Pos** | IIFT Neg | NT | Probable |
| 11/M | Munich, Germany | None | 2013 | Sep 22 | 770 | Pos†† | IIFT Pos | Pos | Confirmed |
| 35/F‡‡ | Berlin | July | 2011–2013 | Sep 23 | 202 | Pos (17.0)‡ | ELISA and IHA Neg§ | Neg | Probable |
| 35/M‡‡ | Berlin | July-August | 2012–2013 | Oct 7 | 359 | Pos (29.0)‡ | ELISA and IHA Neg§ | Neg | Probable |
| 11/M‡‡ | Berlin | July–August | 2012–2013 | Oct 7 | 152 | Borderline (9.0)‡ | ELISA and IHA Neg§ | Neg | Suspected |
| 41/F | Munich | May–June | 2007, 2009–2011 | Oct 10 | 344 | Pos†† | IIFT Pos | Neg | Confirmed |
| 45/F | Montreal, Quebec, Canada | July | None | Dec 5 | 0 | Pos (0.37)§§ | ND | Neg | Suspected |
| 6/F | Berlin | None | 2011–2012 | Dec 22 | 950 | Pos (32.0)‡ | ELISA and IHA Neg§ | Neg | Probable |

*All patients were detected through a search of the GeoSentinel Surveillance Network (*5*). The patient from Montreal had microscopic hematuria; none of the other patients showed signs or symptoms of disease, and their infections were found during screening. IHA, indirect hemagglutination assay; IIFT, indirect immunofluorescence test; Neg, negative; ND, not done; NT, not tested; Pos, positive.
†Suspected, borderline result from 1 serologic testing method; probable, positive result from 1 serologic testing method; confirmed, positive result from 2 serologic testing methods and/or parasite eggs in urine.
‡As determined by using a *Schistosoma mansoni* IgG ELISA (DRG Diagnostics, Marburg, Germany); negative, <9; borderline, 9–11; positive, >11.
§As determined by using an *S. mansoni* adult or egg IgG ELISA; IHA, Cellognost Schistosomiasis H (Siemens, Erlangen, Germany).
¶As determined by using an in-house *S. mansoni* IgG ELISA using egg antigen extract mixed with *S. mansoni* adult worm extract imported from Egypt (positive at an optical density >1).
#As determined by using an in-house IHA with an *S. mansoni* adult worm extract (Fumouze SA, Levallois-Perret, France), with titration and cut-off set at 1/80 (positive at ≥1/160).
**As determined by using an in-house *S. mansoni* cercariae IgG ELISA.
††As determined by using an in-house *S. mansoni* IgG ELISA.
‡‡Familial cluster.
§§As determined by using an in-house *S. mansoni–S. haematobium* combined IgG ELISA (negative, <0.3; borderline, 0.3–0.35; positive, >0.35).

recent infections were acquired after exposure in July or August 2014.

Eosinophilia was recorded for 4 patients (Table). Most diagnoses relied on serologic testing, including the diagnoses for 2 patients who were suspected to have schistosomiasis because of repeated borderline seropositive test results or borderline results plus being part of a familial cluster. Parasite eggs were identified in only 1 patient.

## Conclusions

We document 11 cases of schistosomiasis in international travelers who had freshwater river exposure in Corsica during 2012–2014. Of note, 4 of the persons did not report bathing in the Cavu River (the source of all cases of *S. haematobium* trematode infection among French patients so far), but they did bathe in other Corsica rivers.

Corsica has a population of 316,000 persons, but in 2012, the island was visited by 2.7 million tourists, primarily from mainland France, followed by Italy, Belgium, Germany, and Switzerland (online Technical Appendix Table 2). Local data from the Porto-Vecchio community (≈10,000 inhabitants) confirm the predominance of French nationals among the ≈100,000 tourists who visited the area in 2011; much smaller numbers of tourists visited from other parts of Europe and North America (online Technical Appendix Table 2). Because tourists outnumber residents, it is not surprising that most persons who acquired urinary schistosomiasis in Corsica were tourists (online Technical Appendix Figure). French travelers are not represented in GeoSentinel data because travel across an international border is required for inclusion in the database (*5*). The predominance of German travelers in our cohort may reflect the high proportion of Germans among international travelers to Corsica and the strong representation of German travelers in the GeoSentinel database.

Our report has limitations. All patients identified through the Surveillance Network were asymptomatic at the time of diagnosis. Only 1 traveler had parasitologic proof of infection. In these patients, a diagnosis of schistosomiasis was made on the basis of only 1 positive serologic test result; in some cases, the results were borderline or weakly positive. Serum samples were examined by using in-house

or commercial assays and were not tested side by side in 1 reference laboratory or confirmed by Western blot. We cannot completely exclude that the case definition in our study generated false-positive cases; diagnosis of schistosomiasis in a setting where the disease is not endemic is extremely challenging (*6*). Symptoms of acute schistosomiasis (corresponding to larval migration) may be absent (as in our cases) or nonspecific, but chronic infection (presence of adult worms) due to *S. haematobium* is symptomatic in ≈66% of cases with detectable egg excretion (often of light intensity however) (*7*). In acute and chronic infections in travelers, sensitivity of egg detection is notoriously poor, and serologic test performance is far from optimal.

Schistosomiasis has never been established in Europe. However, sporadic autochthonous cases of human urinary schistosomiasis were reported in Greece, Cyprus, Spain, and Portugal in the 1920s (*8*); the last cases were reported in Portugal in 1965 (*9*). Autochthonous transmission of urinary schistosomiasis to humans has only recently been described in France (*10*). The intermediate host snail, *B. truncatus*, is widely distributed in Africa, the Middle East, and the Mediterranean Basin as far north as Portugal, Spain, Sardinia, and Corsica (*11*). Because the intermediate host is present and climatic conditions are suitable, the risk for autochthonous transmission of *S. haematobium* in the region of Porto-Vecchio was predicted as early as 1928 (*12*). Animal schistosomiasis caused by *S. bovis* was described in cattle in Corsica in 1929; *B. truncatus* snails were identified as the intermediate host. The last cases of animal schistosomiasis in Corsica were documented in 1966 (*13*). The discovery of human cases of schistosomiasis proves that a human–*Bulinus* parasitic cycle exists in Corsica and suggests that a cattle–*Bulinus* cycle may also exist (*13*). Furthermore, hybridization between schistosome species can occur, specifically hybridization of *S. bovis* and *S. haematobium*, as described in Senegal and elsewhere (*14*). Hybridization results in heterosis, thereby producing offspring that have higher fecundity, faster maturation, and a wider intermediate host spectrum.

The situation in Corsica is of significance for One Health medicine and disease epidemiology and, thus, requires a concerted public health and veterinary epidemiologic response. Because a competent intermediate host is present and schistosomes can be imported by migrants and travelers returning (primarily) from sub-Saharan Africa, autochthonous foci of schistosomiasis could become established throughout susceptible Mediterranean areas in southern Europe. The latest data from EuroTravNet indicate that among the >32,000 travelers who returned home ill during 2008–2012, schistosomiasis ranked twelfth among all diagnoses (*15*), and travelers were infected almost exclusively in Africa; none were infected in Europe. We hypothesize that the schistosomiasis outbreak in Corsica began with importation of *S. haematobium* trematodes and subsequent establishment of an autochthonous transmission cycle.

Additional members of the GeoSentinel Surveillance Network who contributed data are Gundel Harms-Zwingenberger (Charité–Universitätsmedizin Berlin, Berlin, Germany), Jakob Cramer (University Clinic Hamburg–Eppendorf), and Brian Ward (McGill University, Montreal, Quebec, Canada).

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# Familial *Trichostrongylus* Infection Misdiagnosed as Acute Fascioliasis

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**To the Editor:** Human fascioliasis, infection with *Fasciola* spp. flukes, is highly pathogenic in both acute and chronic phases and can result in death (*1*). This disease has been recently emerging, in part linked to climate and global changes (*2*). Human *Fasciola* infection has been reported in 5 continents and is related to the disease's wide spread in livestock. Guilan Province in northern Iran is a fascioliasis-endemic area where the largest human epidemics have occurred, together affecting ≈15,000 persons (*3*).

In 2014, 3 sisters (ages 35, 33, and 38) and their 41-year-old brother (patients 1–4, respectively) sought medical care at the same time, all with a 3-week history of symptoms. The patients lived in the Langroud district in Guilan Province, at the Caspian Sea littoral; the sisters lived in the same household with their parents, and the brother lived in a nearby household with his wife and son. Patient 1 had mild abdominal and epigastric pain radiating to her back; onset of abdominal pain and flushing during meals; rigors most prominent at night; severe and voluminous diarrhea intensifying after meals; poor appetite; and urticaria associated with itching on her back, chest, and neck. Patient 2 had epigastric and severe right upper quadrant pain that radiated to her back; severe liver tenderness; weakness; nausea; flatulence; continuous diarrhea; urticarial lesions associated with itching on hands, abdomen, and chest; and a history of backache and pulmonary allergy a few months earlier. Patient 3 had abdominal and epigastric pain, loose defecation, and flatulence; urticaria on the neck; and flushing. Patient 4 had abdominal, neck and shoulder pain; constipation; dyspepsia; severe flatulence; and low-grade fever.

Eosinophil levels for patients 1–4, respectively, were 16,260, 2,640, 13,104, and 3,523 cells/mm$^3$. Results from liver function tests were within reference ranges except for lightly increased alanine aminotransferase (35 IU/L) for patient 2. Sonography of liver, pancreas, and spleen showed no abnormalities. Serologic test results for antibodies to *Fasciola* and *Strongyloides* were negative. All patients denied close contact with herbivorous animals but mentioned regular consumption of fresh vegetables from local markets or from the parents' home garden; the latter had been fertilized with sheep manure a few months before symptom onset.

Acute fascioliasis was diagnosed on the basis of symptoms, weight loss, hypereosinophilia, vegetable consumption, and residence in a high-risk area, all typical associations with this illness (*1*). Absence of fasciolid eggs in stools by Kato-Katz and formalin-ether coprologic methods and lack of sonographic abnormalities were explained by the illness's early invasive phase, and negative serologic results were explained by the immunologic response heterogeneity in fascioliasis (*4*) or antigen deterioration. The patients were treated with a single dose of triclabendazole (10 mg/kg).

One month after treatment, patients returned with the same symptoms and hypereosinophilia. Unexpectedly, patients 1–3 showed *Trichostrongylus* eggs in stools (Figure, panel A). The fourth patient's stool sample was negative. Samples from other family members were analyzed, and 4 (patients' father and mother and the man's wife and son) were shedding *Trichostrongylus* eggs, for a total of 7 patients shedding eggs. *T. colubriformis* and *T. vitrinus* adults (Figure, panels B and C) were identified in feces 24 hours after treatment. This diagnosis was surprising because, although prevalences as high as 71% for *Trichostrongylus* spp. in humans have been described in central and southern Iran (*5*), only sporadic cases have been recently reported.

Infection intensity (24–300 eggs per gram [epg] of feces) correlated with clinical manifestations, indicating light (10–99 epg) to moderate (100–999 epg) severity (*6*). The patient with 24 epg was asymptomatic, and light or moderate cases are known to be asymptomatic (*5*). The acute symptomatology in these patients might be explained by their emaciated and weak conditions. Absence of eggs in the initial analyses may be explained by the long prepatent period (4 months–2 years) and by egg shedding discontinuity in light or moderate infections (*6*–*8*).

The patients fully recovered, and their eosinophilia returned to reference values <1 month after treatment with 1 dose of albendazole (400 mg), followed by mebendazole (200 mg/day for 3 days). One patient who only partially responded was successfully retreated 1 month later.

Trichostrongyliasis and fascioliasis share many epidemiologic and clinical characteristics. *Trichostrongylus* spp. infect livestock worldwide, and human infection has been reported in many countries. Eggs are excreted with feces and then hatch and develop into strongyloid larvae. Humans become infected when ingesting these larvae along with vegetables contaminated by animal feces (*9*). Climate change has been suggested as contributing to the increasing risk for human infection by *Trichostrongylus* spp. (*10*). Trichostrongyliasis patients have symptoms like

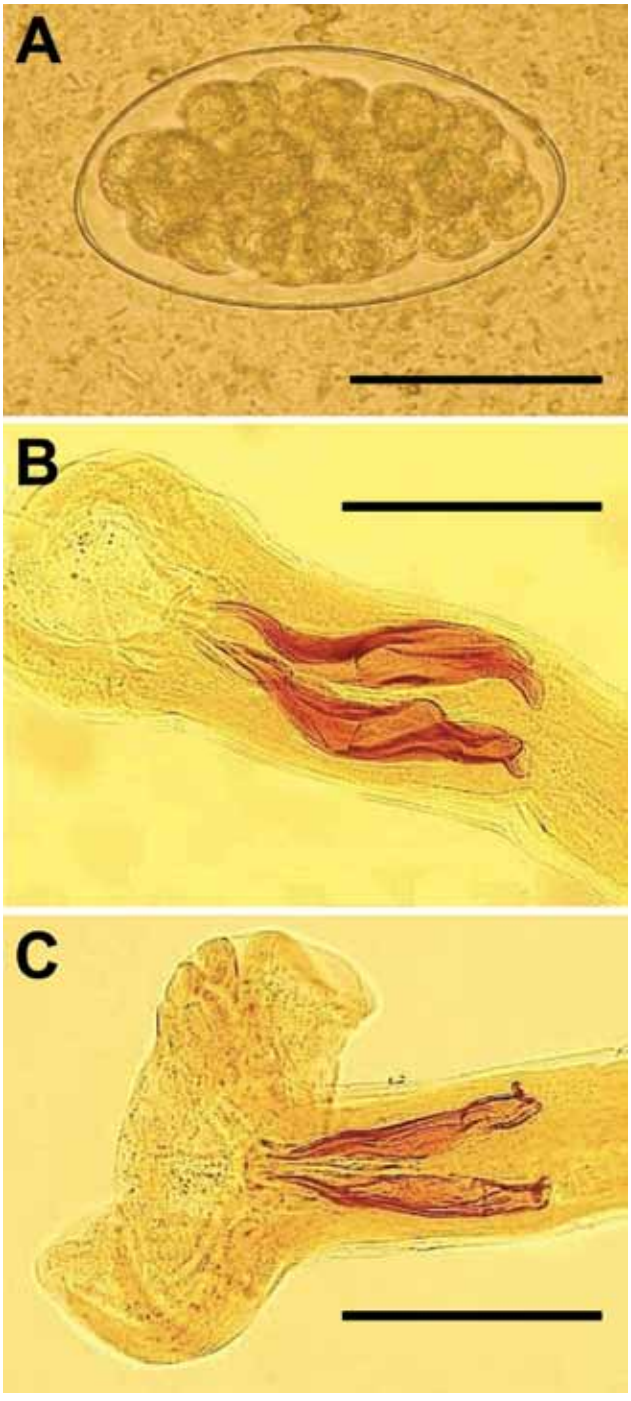


**Figure.** *Trichostrongylus* spp. eggs and nematodes isolated from 1 patient in Guilan Province, Iran, 2014. A) Egg of diameter 87.5 × 48.0 µm obtained from fecal sample of patient by using formalin-ether method. Scale bar indicates 50 µm. B) Bursa copulatrix and spicules (slightly unequal, 135–156 µm long, and boat-shaped with a stepped tip and an outgrowth capping at proximal end) of *T. colubriformis* adult male. Scale bar indicates 100 µm. C) Bursa copulatrix and spicules (equal in size, 160–170 µm long, and straight and tapering sharply at distal end) of *T. vitrinus* adult male. Scale bar indicates 100 µm.

those reported here, although mild eosinophilia may sometimes be the only indication (*6*–*8*). Familial outbreaks related to consumption of fresh vegetables fertilized with sheep and goat manure have been reported (*6*–*9*).

This familial infection cluster highlights the need to consider trichostrongyliasis in patients with suspected fascioliasis in acute or chronic phases without eggs in stools. This diagnosis is especially possible if patients have consumed fresh vegetables fertilized with fresh livestock manure or have had close contact with herbivorous animals.

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# Transmission of *Entamoeba nuttalli* and *Trichuris trichiura* from Nonhuman Primates to Humans

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**To the Editor:** *Entamoeba nuttalli* parasites are frequently found in fecal samples of nonhuman primates (NHPs) in zoos (*1*). This gastrointestinal pathogen is the causative agent of invasive amebiasis in NHPs, which may result in hemorrhagic dysentery, liver abscess or other extraintestinal pathologies, and even death (*2*). *E. histolytica* infection is the cause of invasive amebiasis in humans and can also cause experimentally invasive amebiasis in NHPs (*3*). The host specificity of *E. nuttalli* and *E. histolytica* parasites remains largely unknown. Although molecular analyses indicate that *E. nuttalli* parasites are genetically different from *E. histolytica* parasites (*4*), and hence provide a unique marker to identify zoonotic transmission, molecular tools have not been applied extensively to determine the presence of *E. nuttalli* in humans (*5*). Moreover, studies suggesting transmission have been based on clinical outbreaks in animal caretakers (*2*), and because infections may not always be symptomatic, the results of those studies may underestimate the incidence.

We conducted a study to assess the occurrence of zoonotic transmission of *E. nuttalli* and other gastrointestinal parasites in animal caretakers in 5 zoos in Belgium and the Netherlands. A previous cross-sectional survey (*6*) in these zoos indicated that >80% of the 67 groups of NHPs (40 species, 400 animals, and 1,435 samples) were infected with at least 1 of the 13 gastrointestinal parasites found. In that survey, the most frequently detected parasites were *E. nuttalli* (found in 7 [10.4%] of the 67 groups of NHPs), *Trichuris trichiura* (12 [17.9%]), *Balantidium coli* (14 [20.9%]), and *Giardia duodenalis* (26 [38.8%]). These parasites can cause clinical symptoms in animals and humans. In our study, caretakers of NHPs in each zoo were screened on a voluntary basis for gastrointestinal parasites; animals other than NHPs were also screened (control group). Fecal samples were processed by the acid-ether concentration method and then examined by microscopy to identify the presence of protozoan cysts and helminth eggs (*6*). With the exception of fecal samples from 13 caretakers at 2 zoos, all samples were also processed by using real-time PCR to ascertain the presence of *E. nuttall, E. histolytica,* and *E. dispar* parasites (*7*). *E. nuttalli* can only be detected by PCR targeting the *E. histolytica*–specific repeat; the parasite cannot be detected by a PCR based on the ribosomal small subunit of *E. histolytica*. Both PCRs amplified *E. histolytica* (*8*). Fisher exact test was applied to determine whether there was a difference in parasite infection, as determined by microscopy, between caretakers responsible for NHPs and those caring for animals other than NHPs. An independent physician gave medication to caretakers who were infected with parasites. The study protocol was approved by the Ethics Committee of Ghent University (Belgium; no. 2008/359), Antwerp University (Belgium; no. UA A08 21), and Leiden University Medical Center (the Netherlands; no. 26734).

Fifty-four animal caretakers from 5 zoos participated in the survey; 42 of the caretakers were responsible for NHPs and 12 for animals other than NHPs. Microscopy and PCR results showed that 16 (29.6%) caretakers were infected with ≥1 parasite. We used microscopy to detect cysts of various *Entamoeba* species in fecal samples from 13 (24.1%) caretakers; samples from 4 (7.4%) caretakers were positive for *T. trichiura* eggs. Infections with *Entamoeba* species and *T. trichiura* were observed only in caretakers of NHPs, suggesting that these caretakers are at higher risk of acquiring parasitic infections ($p = 0.03$). PCR detected *E. nuttalli, E. histolytica,* and *E. dispar* parasites in 1, 1, and 3 of 41 caretakers, respectively. We were not able to perform PCR on all fecal samples from 2 zoos (13 caretakers). *E. nuttalli* parasites were detected in a caretaker working in a zoo where *E. nuttalli* infections were prevalent in animals. *E. histolytica* parasites was detected in a caretaker employed in a zoo where *E. nuttalli* infections were not detected in animals. All *T. trichiura* infections were detected in caretakers from 1 zoo, and *T. trichiura* infections were also prevalent in that zoo's animals. None of the caretakers reported gastrointestinal problems.

The presence of *E. nuttalli* and *T. trichiura* parasites in caretakers in zoos where these parasites are also prevalent in NHPs strongly suggests NHP-to-human transmission of these parasites, as was reported for *Blastocystis* parasites (*9*). Genotyping of *E. nuttalli* isolates from NHPs and animal caretakers could be performed for additional confirmation of NHP-to-human transmission of *E. nuttalli* parasites (*10*). The *E. nuttalli*–infected caretaker in this study appeared to be completely asymptomatic. Although *E.*

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*nuttalli* parasites are as virulent as *E. histolytica* parasites in animal models, it remains unclear whether they are as virulent in humans (*4*). We recommend that caretakers of NHPs be screened on a regular basis and be provided with appropriate medications if needed.

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# *Alaria alata* Mesocercariae among Feral Cats and Badgers, Denmark

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**To the Editor:** The digenean trematode *Alaria alata* is considered an emerging zoonotic parasite in Europe because of increased findings in wild boars during *Trichinella* inspection. No human illness caused by *A. alata* mesocercariae (infective larvae) has been reported, but concern remains because the closely related North American species *A. americana* has caused illnesses among humans, including 1 death (*1*).

In Denmark, high prevalence of *A. alata* trematodes in final hosts has been shown (*2*), but limited data on potential paratenic hosts are available. Therefore, samples from 406 domestic pigs, 130 wild boars, 9 badgers, and 99 cats were collected by convenience sampling during October 2013–September 2014. We used pig and wild boar samples from multiple geographic areas of Denmark were leftover tissue samples from ongoing *Trichinella* spp. surveillance. Badgers had died naturally or were hit by vehicles (8 from Jutland, 1 from Zealand) and collected as part of a wildlife monitoring program. Cats (all from Zealand) were either feral (n = 92) or domesticated (n = 7) and had been euthanized as part of a national control program. Carcasses were necropsied in our laboratory; we collected 30 g of tissue samples according to Riehn et al. (*3*). All samples were analyzed by the modified *A. alata* mesocercariae migration technique (*3*). In brief, the sample was cut into ≈0.5-cm edge pieces, wrapped in gauze, and suspended for 90 min by 2 wooden sticks in a conical glass with ≈300 mL of water (46°C–48°C). Approximately 15 mL of sediment was collected from the bottom of the glass by suction by using a glass pipette and examined by microscopy (magnification ×20).

*A. alata* mesocercariae were isolated from 3 cats and 6 badgers (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/20/10/14-1817-Techapp1.pdf). All 3 cats were female (2 pregnant, 1 lactating); prevalence was significantly higher in pregnant or lactating females (3/12) than other intact females (0/24) (p = 0.031 by Fisher exact test). This finding might be related to increased exposure because an increase in predation by the cats during pregnancy and lactation to meet higher protein and energy demand. However, because *A. marcianae* mesocercariae can be

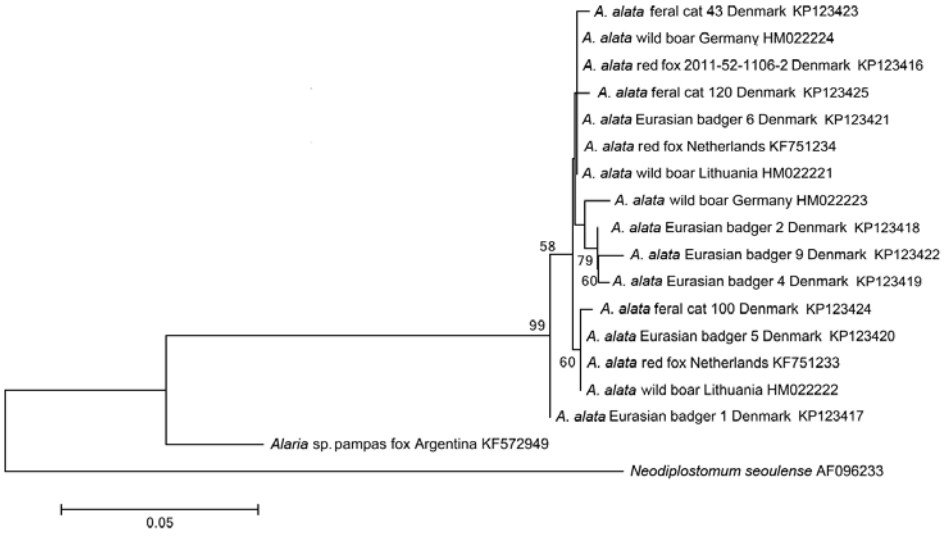


**Figure.** Neighbor-joining phylogenetic tree of *Alaria alata* isolates based on the analysis of partial mitochondrial cytochrome c oxidase subunit 1 gene sequences (332 bp). Bootstrap values are indicated to the left of the nodes and are based on 10,000 replicates. GenBank accession numbers are listed to the right. Scale bar indicates base substitutions per site.

transmitted through milk in cats (*4*), lactating females may also be predisposed to an increased chance for *A. alata* mesocercariae reaching their offspring. Examination of the intestines of all cats by sedimentation and counting technique (*5*) revealed no *A. alata* adults. Although *A. alata* adults have been found in cats in Uruguay (*6*), reports from Europe are lacking, and thus it is still uncertain whether cats can act as amphiparatenic or final hosts. Natural infection of cats with other *Alaria* spp. has been reported in the United States (*7*), indicating biologic differences among *Alaria* spp.

Zoonotic risk for *A. alata* infection through ingestion of cat meat is probably minimal in Europe but may be important in Asia and South America, where cats are occasionally consumed. Badgers are, however, sometimes consumed as game meat or road kill meat in Europe. In Russia, 10.6% of trichinellosis outbreaks during 1998–2002 were caused by consumption of badger meat (*8*). Thus, the zoonotic potential of infections in this animal, although a protected species, should not be ignored.

Negative findings in domestic pigs and wild boars in this study may reflect underestimation because those samples were below the recommended 30 g and often taken from sites that are not typically infected with mesocercariae (*3*). A follow-up study with better sampling strategy would be of value to determine the risk for *A. alata* transmission from domestic pigs and wild boars.

Identification of isolated mesocercariae was confirmed by PCR and sequencing of a fragment (332 bp) of the mitochondrial cytochrome c oxidase subunit 1 gene (*cox*1) (*9*). By neighbor-joining analysis (*10*), the consensus *cox*1 sequences were compared with the trematode *Neodiplostomum seoulense* (outgroup), 1 *A. alata* isolate from a Danish red fox, and all 7 *cox*1 sequences of *Alaria* spp. available in GenBank as of October 2014. (Sequences from this study have been deposited into GenBank under accession nos. KP123417–KP123422 [badgers] KP123423–KP123425 [feral cats].) The inferred phylogenetic tree (Figure) showed marked genetic variation among *A. alata* isolates from Denmark and other parts of Europe but no apparent separation of most *A. alata* isolates from Europe based on host species or country, except for that from badger 1 (online Technical Appendix Table 2). This animal originated from Northern Jutland, where host and parasite populations are geographically isolated by a large fjord separating the region from the rest of the country. The marked genetic variation within *cox*1 sequences suggests the usefulness of this marker, but additional genetic markers should be included in future studies to explore the genetic flow of *A. alata* within natural hosts.

In conclusion, *A. alata* mesocercariae seem to favorably infect pregnant or lactating cats, thereby increasing the chance of vertical transmission. Further, detection of *A. alata* infection in numerous badgers suggests potential high zoonotic risk associated with ingestion of such exotic meat. These results should, however, be interpreted with caution because of the small sample size and unknown efficacy of the modified *A. alata* mesocercariae migration technique.

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# Human Infections with *Pseudoterranova cattani* Nematodes, Chile

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**To the Editor:** Anisakidosis is an emerging foodborne zoonosis caused by nematode larvae of the Anisakinae subfamily, which includes the genera *Anisakis*, *Pseudoterranova*, and *Contracecum* (*1*). In natural cycles, anisakid larvae are transmitted to marine mammals or piscivorous birds when they eat raw saltwater fish or squid. In the human incidental host, larvae attach to the mucosa of the gastrointestinal tract, causing clinical features ranging from asymptomatic carriage to severe abdominal pain with complications, such as gastric perforation (*2*). Microscopical diagnosis is hampered by the lack of distinguishing morphologic characteristics in larval stages (*1*). Recently, molecular genetic techniques have shown that the main species, *Anisakis simplex* and *Pseudoterranova decipiens*, are in fact species groups with distinct geographic and biologic characteristics (*3*,*4*). The *P. decipiens* complex consists of at least 6 sibling species (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/10/14-1848-Techapp1.pdf). We report 4 human infections with *P. cattani* diagnosed during 2012–2014.

The case-patients were adults 22–59 years of age; 2 were female, and all lived in Santiago, Chile. Additional anamnestic and clinical data were available for 3 patients: all spontaneously regurgitated the parasites without having other gastrointestinal complaints. All 3 reported eating ceviche, a dish made of raw marine fish marinated in lemon juice. One patient reported a tingling sensation and coughs before the expulsion of a highly motile larva (Video, http://wwwnc.cdc.gov/article/21/10/14-1848-V1.htm). This patient was awaiting oral surgery after a bicycle accident and had eaten the last raw fish dish 2 weeks previously. Initially, parasites were identified by morphologic criteria. Larvae were 20 mm long, were of whitish to reddish color, and had 3 anterior lips (online Technical Appendix Figure 1). Because of the presence of an anteriorly directed cecum (online Technical Appendix Figure 2), they were assigned to *Pseudoterranova* species.

For further molecular identification, DNA samples were extracted by using a DNeasy Blood and Tissue Kit (QIAGEN K.K., Tokyo, Japan). The rRNA gene containing 2 internal transcribed spacer (ITS) regions was amplified by PCR using primers NC5 and NC2, as previously described (*5*). PCR products were sequenced by using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Inc., Foster City, CA, USA) on an automated sequencer (ABI3100, Applied Biosystems). Sequence similarities were determined by a BLAST search of DDBJ (http://blast.ddbj.nig.ac.jp/top-j.html). The GENETYX-WIN program version 7.0 (Software Development Co., Tokyo, Japan) facilitated sequence alignment and comparison. Within the 4 ITS sequences of amplicons obtained, all were 100% identical, and alignment with the other *P. cattani* sequence differed only in 1 nt. ITS sequences of 2 isolates are available in GenBank (accession nos. KF781284 and KF781285). All *P. cattani* sequences showed a previously described

[1]These authors contributed equally to this article.

**Table.** Alignment (comparison) of nucleotide sequences of the ITS1 gene of *Pseudoterranova cattani* and the Chilean specimen and *P. decipiens**

| Isolate | ITS1 sequence at 240–270 nt | GenBank accession no. |
|---|---|---|
| Pc1 | CTCTGTT----------AACGCAGAGT | AJ413981 |
| CL#3 | CTCTGTT----------AACGCAGAGT | KF781284 |
| PdCa1 | CTCTGTTTTGGTTTCAACGCTAACGCAGAGT | AJ413979 |

*CL#3, specimen from Chile; Pc1; ITS, internal transcribed spacer. Pc1, *P. cattani*; PdCa1, *P. decipiens*.

deletion of ≈14 bases (Table), which is not observed in other members of the *P. decipiens* species complex (*5*).

This study identified *P. cattani* as a parasite capable of infecting humans. The definitive natural host of this parasite is the South American sea lion, *Otaria byronia*. At least 4 species of coastal fish were described as intermediate or paratenic hosts, including popular Chilean food fish species, such as *Merluccius gayi*, *Genypterus maculatus*, and *Cilus gilberti* (*6*). The spectrum of species causing human pseudoterranovosis is uncertain because most cases were reported as *P. decipiens* (sensu lato) or *Pseudoterranova* sp. Only recently, 1 case of *P. azarasi* infection has been documented in a patient from Japan (*7*). Although comparative studies are lacking, *Pseudoterranova* larvae seem to be less invasive and cause milder symptoms than *Anisakis* larvae (*2*,*8*). In the cases reported here, larvae were spontaneously expelled without further symptoms, except in 1 patient who reported the typical feature of noninvasive pseudoterranovosis, also described as "tingling throat syndrome" (*8*), a foreign body sensation accompanied by cough and retching. In Chile, ≈30 human cases have been reported, all diagnosed as *P. decipiens* or *Pseudoterranova* sp. by morphologic criteria (*9*,*10*). Most patients described mild oropharyngeal complaints and cough. More severe manifestations similar to parasitic pharyngitis caused by *Fasciola hepatica* or *Linguatula serrata* seem to be absent, although 1 patient had symptoms of asphyxia (*9*). The extent to which these cases in Chile were caused by *P. cattani* is uncertain because molecular diagnosis was not performed. The length of stay and location within the human gastrointestinal tract of *Pseudoterranova* larvae are unknown, but as indicated by 1 case in our report, lack of symptoms for up to 2 weeks is possible.

These cases demonstrate that *P. cattani* is an incidental human parasite causing oropharygeal pseudoterranovosis. To better understand the epidemiology and clinical relevance of these emerging fishborne zoonotic infections, molecular diagnostic techniques need to be more widely applied, especially in regions where raw fish is part of the regular diet, such as in many parts of South America.


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# Severe Sepsis Caused by California Serogroup Orthobunyavirus

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**To the Editor:** The California serogroup (CAL) of orthobunyaviruses, including Jamestown Canyon virus (JCV) and La Crosse virus (LACV), can cause neuroinvasive disease in humans (*1*–*3*). The epidemiology and clinical spectrum of LACV are well described (*3*), whereas less is known about JCV.

JCV is distributed throughout temperate North America, and its transmission cycle involves ungulates and several mosquito genera (*4*–*6*). The first human case of JCV disease in Minnesota, USA, was reported in 2013 (*7*), although serologic evidence of the virus had been previously documented in the white-tailed deer population (*8*). Of the 22 human cases reported to the Centers for Disease Control and Prevention in the United States during 2013, 15 were neuroinvasive (*7*). However, given the lack of widespread testing availability and the few reported cases, the actual clinical spectrum of JCV infection, unlike that of LACV, remains unclear. We report a case of severe sepsis in an adult with antibodies against CAL and suspected JCV disease.

A 62-year-old man from Waseca County, Minnesota, who had type 2 diabetes mellitus, obesity, migraine headaches, depression, and anxiety, had been vacationing for 1 week at his cabin in Carlton County, Minnesota. On July 9, 2014, he experienced diffuse myalgia, arthralgia, and headache. He sought treatment July 10 at a local urgent care facility for fever, rigors, sweats, weakness, dizziness, poor appetite, and mild shortness of breath, but he denied having cough, chest pain, gastrointestinal symptoms, neck stiffness, and photophobia. He described extensive mosquito exposure the prior week. On physical examination, temperature was 38.8°C, and he had multiple skin lesions consistent with mosquito bites. A 5-cm, lacy, petechial skin rash was observed on the right inner thigh. Chronic venous stasis changes of the lower extremities were noted, and a faint systolic cardiac murmur was heard. Peripheral leukocyte count was $11.2 \times 10^9$ cells/L, and electrolyte levels were within reference ranges. Serologic screenings for likely infectious agents were negative (Table). Empiric doxycycline was initiated.

Two days later, he experienced worsening myalgia, arthralgia, fevers, profuse sweating, nausea, and vomiting and visited a local emergency department. Mild confusion was noted on examination. Temperature was 37.9°C, and blood pressure was 69/32 mm Hg, with minimal improvement despite intravenous fluid administration. Peripheral leukocyte count was $16.2 \times 10^9$ cells/L, serum creatinine and blood-urea nitrogen levels were elevated at 1.7 and 37 mg/dL, respectively, and liver enzyme and lactate levels were normal. Results of a chest radiograph were unremarkable. Dopamine and broad-spectrum antibiotic drugs were initiated, and he was transferred to Mayo Clinic (Rochester, Minnesota) in the intensive care unit. At that time, no confusion was noted, nor signs or symptoms of neuroinvasion that would have warranted cerebrospinal fluid examination or neuroimaging.

Hypotension resolved overnight, and leukocytosis and serum creatinine levels improved. A low-grade fever of 38.0°C was documented in the evening of July 14. Because the overall etiology of the illness remained unclear, a computed tomography scan of the abdomen and pelvis was performed to investigate potential intraabdominal sources of sepsis, but results were unrevealing. By hospital day 5, blood cultures remained negative, leukocytosis resolved, and creatinine levels normalized. Antibiotic drugs were discontinued, and the patient was discharged with a 2-week course of oral doxycycline. Soon after discharge, an acute-phase serum specimen was positive for a CAL orthobunyavirus (Table).

During the next 3 weeks, the man sought treatment multiple times as an outpatient for diarrhea, lightheadedness, dizziness, headache, fatigue, difficulties concentrating, and subjective memory loss. These symptoms gradually resolved over 2 months. Convalescent-phase serum samples were drawn 3 weeks after dismissal and showed 32-fold increased antibody titers against CAL. The Minnesota Department of Health was notified, and plaque reduction neutralization testing was ordered to determine the specific virus. Increases in the neutralizing antibody titers against JCV and LACV were noted; although the absolute titer was greater for JCV (Table), a final determination of which virus caused the patient's illness could not be made. Neutralizing antibodies in the acute-phase specimen in the absence of IgM suggests that the patient was previously exposed to an orthobunyavirus.

Antibody cross-reactivity between closely related arboviruses such as LACV and JCV is not unusual, and similar antigenic sin has been described with other orthobunyaviruses (*9*). Epidemiologic factors, including time of year, location of mosquito exposure, and the predilection of LACV disease for children, point to JCV infection in this case, although it cannot be confirmed.

Previous reports of JCV disease have focused on the ability of this virus to cause asymptomatic infection,

**Table.** Pathogen testing in patient with undifferentiated severe sepsis, Minnesota, USA, 2014*

| Pathogen tested | Serum sample | |
|---|---|---|
| | Acute phase | Convalescent phase |
| HIV-1/-2 | Ag/Ab screen negative | |
| *Leptospira* | IgM/IgG negative | |
| *Cryptococcus* | Antigen screen negative | |
| *Anaplasma phagocytophilum* | IgG <1:64, PCR negative | |
| *Ehrlichia chaffeensis* | IgG <1:64, PCR negative | |
| *Ehrlichia ewingii/canis* | PCR negative | |
| *Ehrlichia muris*–like | PCR negative | |
| *Borrelia burgdorferi* | Ab screen negative | Ab screen negative |
| *Babesia microti* | IgG <1:64, PCR negative | |
| *Babesia duncani* | PCR negative | |
| *Babesia divergens* strain MO-1 | PCR negative | |
| West Nile virus | IgM/IgG and PCR negative | IgM/IgG negative |
| Eastern equine encephalitis virus | IgM/IgG <1:10 | IgM/IgG <1:10 |
| Western equine encephalitis virus | IgM/IgG <1:10 | IgM/IgG <1:10 |
| St. Louis encephalitis virus | IgM/IgG <1:10 | IgM/IgG <1:10 |
| California serogroup virus | IgM ≥1:10,† IgG 1:10 | IgM 1:80, IgG 1:320 |
| Powassan/tick-borne encephalitis virus‡ | IgM negative | |
| La Crosse virus‡ | IgM negative, PRNT <10 | PRNT 320 |
| Jamestown Canyon virus‡ | IgM negative, PRNT 160 | PRNT 10,240 |

*Positive control value for PRNT testing was >1,280. Ag, antigen; Ab, antibody, Ig, immunoglobulin, PRNT, plaque reduction neutralization testing.
†Additional dilution of result not performed.
‡Testing performed at the Arboviral Diseases Branch Laboratory, Centers for Disease Control and Prevention, Fort Collins, Colorado, USA.

nonspecific mild febrile illness, or severe neuroinvasive disease (*4*,*7*,*10*). This case illustrates a suspected JCV infection causing undifferentiated severe sepsis, which has not, to our knowledge, been previously reported. Initial suspicion for acute neuroinvasive disease was low, and neurologic imaging and cerebrospinal fluid sampling were not performed. We recommend that testing for CAL (and specifically for JCV) infection should be strongly considered in the setting of severe sepsis in adults with substantial exposure to mosquitoes and no other identifiable source of sepsis.

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## Correction: Vol. 21, No. 7

An additional case report publication was found after publication of Disseminated Infections with Talaromyces marneffei in Non-AIDS Patients Given Monoclonal Antibodies against CD20 and Kinase Inhibitors (J.F.W. Chan al.). An addendum and reference have been added to the article online (http://wwwnc.cdc.gov/eid/article/21/7/15-0138_article).

# Bloody Diarrhea Associated with Hookworm Infection in Traveler Returning to France from Myanmar

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**To the Editor:** Human hookworm infections are commonly caused by 2 anthroponotic species, *Necator americanus* and *Ancylostoma duodenale*. However, *A. ceylanicum*, a zoonotic hookworm of canids and felids, is emerging as the second most common human hookworm in Southeast Asia (*1–4*). Two haplotypes of *A. ceylanicum* hookworm have been identified, 1 specific to humans and 1 specific to humans, dogs, and cats (*4,5*). We report a case of patent enteric *A. ceylanicum* hookworm infection in a man from France who had visited Myanmar.

In December 2014, a 33-year-old man with no medical history sought care in France after 3 weeks of fever, vomiting, dyspnea, bloody diarrhea, and weight loss (7 kg). He had returned from a 3-week trip to Myanmar 1 month earlier. Two days after his arrival in Myanmar, he had pruritic erythematous macules on the buttocks after sitting in a public park in Rangoon while wearing short pants; this sign was followed by a dry cough.

Laboratory data showed leukocytosis (17.43 × $10^9$ cells/L) with hypereosinophilia (55%) and a hemocrit of 56.1%. Direct examination of hemorrhagic stool showed numerous Charcot-Leyden crystals (CLCs) and 150–200 eggs/g feces of unembryonated hookworm ova (mean size 57.6 × 38.4 μm) (Figure, panel A). Rhabditiform and filariform larvae were isolated by stool culture (Figure, panel B). On the basis of clinical history and data suggestive of eosinophilic enteritis, which is uncommon in patients infected with parasites adapted to humans, a zoonotic hookworm species was suspected.

For species identification, DNA was extracted from larvae by using the DNeasy Tissue Kit (QIAGEN, Hilden, Germany) at the Faculty of Veterinary and Agricultural Sciences, University of Melbourne (Parkville, Victoria, Australia) and subjected to PCR specific for the ribosomal internal transcribed spacer region of hookworms (*1*). Testing was conducted at the University of Melbourne because this institution has the technical expertise for identifying hookworm. In addition, haplotype characterization was performed by using PCR specific for the mitochondrial cytochrome oxidase-1 gene (*4*). Bidirectional DNA sequences of PCR products (Macrogen Inc., Seoul, South Korea) were analyzed by using Finch TV 1.4.0 (Geospiza Inc., Seattle, WA, USA). The ribosomal internal transcribed spacer region had 100% sequence identity with an *A. ceylanicum* sequence in GenBank (DQ381541). Neighbor-joining analyses with MEGA 4.1 (http://www.megasoftware.net) clustered the isolate within the *A. ceylanicum* haplotype specific for animals and humans (*4*).

By the third day of albendazole therapy (400 mg/d), clinical improvement was observed and stool specimens were negative for hookworm ova. However, eosinophilia

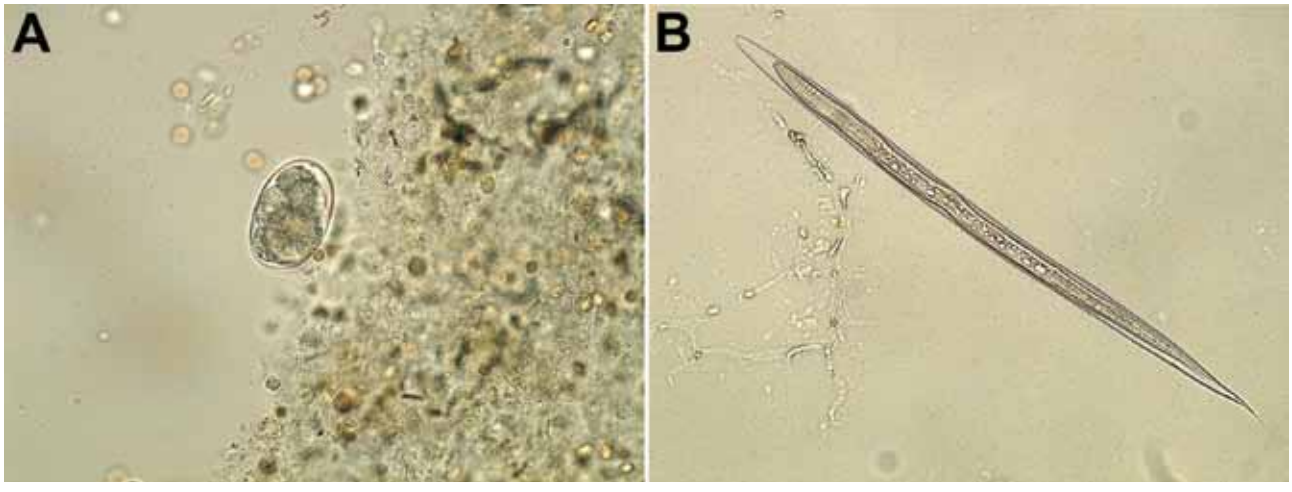


**Figure.** *Ancylostoma ceylanicum* hookworm isolated from a French tourist returning from Myanmar. A) Ova and blood cells in fecal specimen. B) Filiform larvae after stool culture. Original magnifications ×40.

(59%) was persistent. Three months later, the patient was hospitalized because of diarrhea and abdominal pain. CLCs and hookworm ova were observed, and the patient received a second course of albendazole.

*A. ceylanicum* hookworm was unequivocally identified by using molecular methods as the etiologic cause of the original signs and symptoms for this patient. In Asia, *A. ceylanicum* hookworm is reported at a prevalence of 62%–92% in stray dogs and cats (*3*) and environmental contamination of the public park by dog feces was the probable source of infection for this patient.

*A. ceylanicum* hookworms have been experimentally shown to develop to patency in humans within 26–35 days (*6*), but abdominal symptoms and eosinophilia may occur earlier (within 21 days). Natural human infections have been described in most regions where *A. ceylanicum* hookworm is endemic to animals, but clinical and pathologic data are scarce. In Taiwan and Malaysia, this hookworm was visualized in the mid-jejunum in patients with acute, severe abdominal pain and nausea and in terminal ileum in patients with anemia caused by chronic blood loss, nausea, and melena (*7*–*9*).

In all cases, leukocytosis with eosinophilia (22%–50%) was observed. In the patient we describe, transdermal infection causing cutaneous larva migrans was followed by development of eosinophilic enteritis within a 2-week period. Enteric signs were similar to those observed in previous cases in Taiwan. A 3-day course of benzimidazole is the anthelminthic drug of choice. However, clinicians must be aware of possible relapse, potentially caused by failure of the adulticidal drug to kill developing larvae before full patency is reached (*3,8,9*).

Differentiation of larvae and eggs of anthroponotic and zoonotic *Ancylostoma* spp. is difficult. Definitive diagnosis relies on detailed morphologic identification of adult worms or molecular identification of adults, eggs, or larvae in stool specimens. Thus, human infection in travelers returning from parasite-endemic regions is likely to be misdiagnosed as an anthroponotic hookworm species. Clinically, the presence of hookworm ova and an unusually high number of CLCs and eosinophils in stool should alert clinicians to the possibility of an infection with *A. ceylanicum* hookworm.

*A. ceylanicum* hookworm is reported primarily in tropical climates; the possibility of spread in temperate countries remains low because development of filariforme larva requires high temperatures and a moist environment. However, recent climate changes, coupled with poor sanitary conditions, could promote emergence of tropical species, and recently, rare cases of autochthonous hookworm-related cutaneous larva migrans have been reported in Europe (*10*).

This report highlights the risk for zoonotic ancylostomiasis in travelers visiting countries to which *A. ceylanicum* hookworm is endemic among animals. It also emphasizes the usefulness of copromolecular techniques for species-specific diagnosis.

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# Vaccine-Derived Polioviruses Not Detected by Global Surveillance Screening Assay

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**To the Editor:** The Global Polio Laboratory Network (GPLN) and the World Health Organization's Polio Eradication Initiative (GPEI) accord high priority to detecting all vaccine-derived polioviruses (VDPVs) because they are neurovirulent and have the potential to cause outbreaks of poliomyelitis and establish poliovirus circulation. In patients with immunodeficiency diseases, persistent infections may become established with live oral poliovirus vaccine (OPV) and develop into VDPVs (*1*). Laboratories of the GPLN use standard procedures for virus isolation, identification, and intratypic differentiation (*2*). The real-time reverse transcription PCR (rRT-PCR) VDPV screening assay became available to the GPLN in 2009. Poliovirus isolates that do not become amplified in the VDPV assay are subjected to complete sequencing of viral protein (VP) 1. VDPVs are isolates of Sabin OPV origin that have incorporated ≥6 nucleotide substitutions (Sabin2) or ≥10 nucleotide substitutions (Sabin1 and Sabin3) in the VP1 region. The VDPV assays were found to be 100% specific for all 3 poliovirus types, 100% sensitive for Sabin1 and Sabin3, and 76% sensitive for Sabin2 (*3*). Among all cases of circulating VDPV infection reported globally from 2000 to 2013, 10.95%, 97.1%, and 1.8% of cases were caused by types 1, 2, and 3, respectively (*4*).

To mitigate the risk for infection with VDPV type 2, GPEI envisions a simultaneous global switch from trivalent OPV to bivalent OPV (Sabin1 and Sabin3)—that is, withdrawal of Sabin2—beginning in April 2016 (*5*). Any type 2 polioviruss detected thereafter will need characterization. Here we report VDPV isolates that escaped detection by the VDPV screening assay used in the GPLN.

Type 3 VDPV was identified in a 1.2-year-old girl with onset of acute flaccid paralysis (AFP) on January 2008. Sabin3 was isolated from stool sample 1 (R46064), collected 11 days after onset of paralysis. Stool sample 2, collected on the 13th day after paralysis, was negative for virus. R46064 was reported as "Sabin-like" by the intratypic differentiation tests (ELISA and conventional RT-PCR) used in the GPLN in 2008 (*6*,*7*). The patient had residual weakness compatible with paralytic poliomyelitis; therefore, the isolate was characterized in detail. Complete genome sequence of R46064 showed that the major attenuation sites reverted to wild type at nt 472 (U→C) in the 5′ untranslated region (UTR) and nt 2034 in VP3 (U→C). The capsid region contained 18 nt substitutions, of which 12 were in the VP1 region (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/21/10/15-0702-Techapp1.pdf). Seven amino acid changes occurred, including 2 at the antigenic site NAg1 (online Technical Appendix Figure 1). The isolate was a recombinant with species C enterovirus in the noncapsid region. R46064 was a VDPV3 isolate by definition. Investigations showed that VDPV3 was not a part of any outbreak.

R46064 produced Sabin3-like results in the VDPV screening assay. R46064 gave false-negative test results because the isolate had a Sabin3 vaccine sequence in the regions corresponding to the probe and primers of the VDPV assay (online Technical Appendix Figure 2).

Type 2 VDPV was found in an immunocompetent girl, 3.5 years of age, in March 2014. Sabin2 was isolated from 2 stool samples collected 5 and 8 days after onset of AFP. Sabin2, isolated from stool sample 1 (R93150), was amplified in the VDPV screening assay (reported as Sabin2-like); the isolate from stool sample 2 (R93152) failed to become amplified (reported for sequencing).

VP1 sequencing of R93152 showed 6 nt substitutions; therefore, it was reported as VDPV2. R93150 was also sequenced to find out whether it contained the Sabin2 homotypic mixture. VP1 sequence of R93150 showed 6 nt substitutions and no evidence of mixed bases. Substitution was not found at VP1 nt 427/428, the main target of the VDPV screening assay.

Complete genome sequence analysis revealed that both isolates contained reversion of the major attenuating site at nt 481 in the 5′ UTR. The genomes of both isolates showed no recombination. Both isolates showed 15 nt substitutions in the capsid region, when compared with Sabin2 (online Technical Appendix Table); only 5 substitutions were common to both isolates. R93150 had 4 aa changes; 1 change was at the antigenic site NAg2. R93152 showed 6 aa changes; 2 changes were at NAg3a and NAg3b (online Technical Appendix Figure 1). Thus, VDPVs of 2 distinct VDPV2 lineages were excreted by the patient; 1 isolate was identified as VDPV2, but the other was missed. Although the patient was identified as having VDPV2 infection by the current algorithm, if special interest had not been taken to characterize both isolates, we would not have detected the VDPV2 that produced false-negative results in the screening assay.

The occurrence of VDPV2 and VDPV3 as described above may be rare. However, GPLN laboratories are unlikely to detect VDPV strains that produce false-negative results in the VDPV screening assay. False negatives are of greatest concern to the GPEI because they could impede timely detection of VDPV infections (*3*,*8*). Our results

point out the need for reporting and inventorying VDPVs that give a false-negative reaction in the screening assay. This action would help clarify how to further refine the screening assays.

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# Association of Necrotizing Wounds Colonized by Maggots with *Ignatzschineria*–Associated Septicemia

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**To the Editor:** *Ignatzschineria* is a recently described genus of bacteria that have been rarely implicated in human disease (*1*–*3*). We report a patient in France with septicemia caused by *I. ureiclastica*.

In October 2013, a 69-year-old man was found unconscious in a forest close to Tours in the Loire Valley, France. The patient had hypotension with auricular fibrillation complicated by cardiorespiratory arrest and was admitted to the general intensive care unit of Tours University Hospital. He also had cyanosis of the extremities, a necrotic skin lesion on the right shoulder, and a large number of maggots around the genital organs. Empiric treatment with ceftriaxone was initiated. Blood cultures on admission revealed several microbes: *Enterococcus faecalis*, *Enterobacter cloacae*, *Providencia stuartii*, *Corynebacterium* spp., and a gram-negative bacillus resembling *Pseudomonas*. This bacillus was sensitive to all β-lactams, aminosides, fluoroquinolones, colistin, and trimethoprim/sulfamethoxazole but was resistant to fosfomycin. Ten days after admission to the hospital, the patient was found dead in his bed from no evident cause, despite recent improvement of his clinical state. No autopsy was conducted.

The unidentified gram-negative bacillus was an oxidase-positive strict aerobe. The 16S rRNA and *gyrB* genes were amplified and sequenced (*4*,*5*). The 897-bp 16S rRNA sequence obtained for the bacterium was 99% identical to sequences from *I. larvae* type strain L1/68T (GenBank accession no. AJ252143) and *I. ureiclastica* type strain FFA3T (GenBank accession no. EU008089). The 973-bp *gyrB* sequence of the isolate was 96% similar to the sequence of *I. ureiclastica* type strain FFA3T (GenBank accession no. FJ966120) and 92% with *I. larvae* type strain L1/68T (GenBank accession no. FJ966121). The 16S rRNA and *gyrB* sequences (GenBank accession nos. KR184134 and KR184135) were compared with

those of all members of the genus *Ignatzschineria* and with those of several species belonging to the class Gammaproteobacteria. Two phylogenetic trees were deduced by the neighbor-joining method (Figure).

The genus *Ignatzschineria*, which is the revised nomenclature for *Schineria*, was first described in 2001. It comprises 3 species: *I. larvae*, *I. indica*, and *I. ureiclastica* (*6*–*8*), and belongs to the family *Xanthomonadaceae*, class Gammaproteobacteria,. All 3 species have been isolated from larvae *Wohlfahrtia magnifica* flies (*9*), which are found in Europe, Asia, and North Africa and cause myiasis in several animal species but rarely in humans. *Ignatzschineria* spp. is the dominant species in the anterior portion of the digestive tract in larvae, together with *Providencia* (*9*). *Providencia* was also found in blood cultures from this patient. Cases of *I. larvae* and *Ignatzschineria* sp. bacteremia were reported in France: 1 in a homeless patient (*2*) and the other in a patient with type 2 diabetes (*1*), both with a foot wound invaded by maggots. Three cases of *I. indica* infection were recently described in the United States: 2 cases of bacteremia and 1 urinary tract infection (*3*). These 3 cases were clearly associated with fly larvae infestations and myiasis.

The presence of *I. ureiclastica* in the blood cultures of the patient reported here and the presence of bacteria from the same genus in 4 other cases of bacteremia suggest an association between *Ignatzschineria* bacteremia and wounds infected by maggots in patients with poor hygiene. Systematic blood cultures should therefore be conducted for such patients. The epidemiologic importance of *Ignatzschineria* spp. might have been underestimated because of the presence of other microbes in samples and identification difficulties, which in some cases might have led to a conclusion of simple contamination.

The species of fly larvae found in wounds and the bacteria transmitted appear to differ among geographic regions. In France, *I. larvae* and *I. ureiclastica* are the species associated with the *W. magnifica* fly, which is present in Europe, Asia, and North America. In the United States, the 3 human infections reported were all caused by *I. indica* and seemed to be associated with larvae of the *Phaenicia sericata* fly, found throughout the world. A geographic specificity of *Ignatzschineria* spp. linked to the geographic distribution of fly larvae is therefore remarkable.

The larvae used in maggot therapy are "sterile" larvae of the *P. sericata* fly. A possible risk for infection with *Ignatzschineria* exists with larval therapy, especially with *I. indica*.

The pathogenic power of *Ignatzschineria* spp. remains to be demonstrated. However, a wound invaded by maggots seems to be strongly associated with the presence of *Ignatzschineria* spp. in clinical samples, with the possibility of a specific geographic distribution of the species implicated. Clinicians and microbiologists should be aware of the possibility of invasive *Ignatzschineria* infections in presence of maggots in patients with poor hygiene and should check specifically for this bacterium.

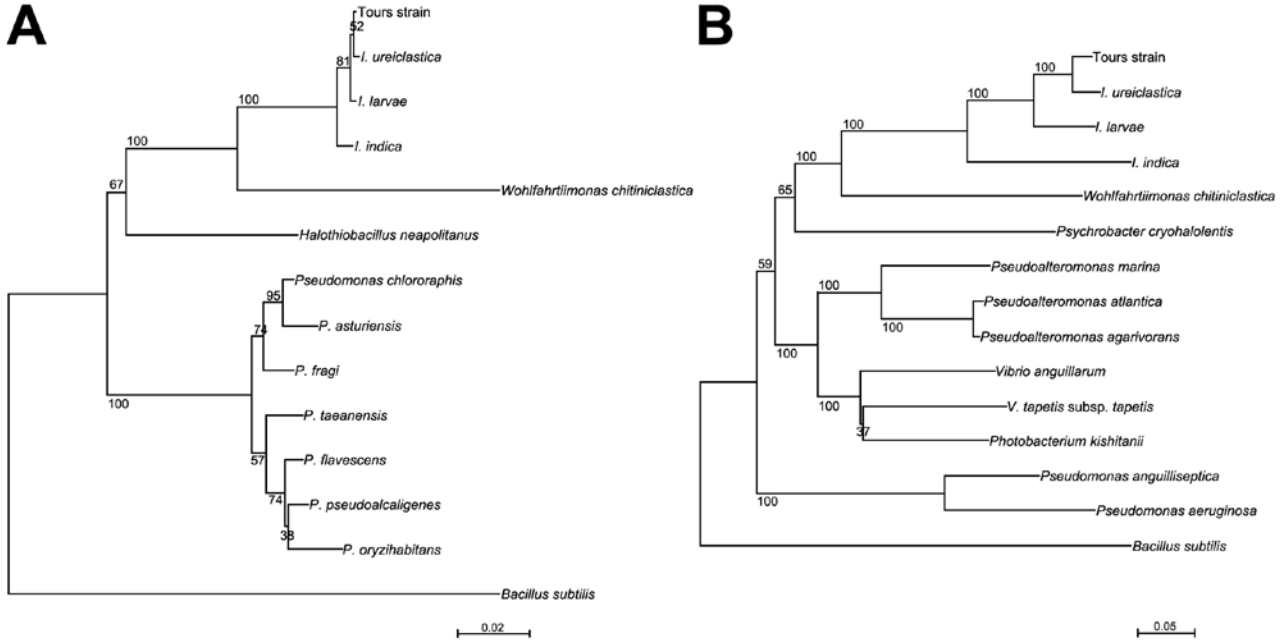


**Figure.** Phylogenetic trees showing relationships between the clinical isolate identified in this study ("Tours strain") and type strains of members of the genus *Ignatzschineria*. A) Relationships among 16S rRNA sequences of "Tours strain" (GenBank accession no. KR184134) and *Ignatzschineria* strains; scale bar represents 2% differences in nucleotide sequence. B) Relationships among *gyrB* sequences of "Tours strain" (GenBank accession no. KR184135) and *Ignatzschineria* strains; scale bars represent 5% differences in nucleotide sequence. *Bacillus subtilis* was included as an outgroup organism. Numbers at branch nodes are bootstrap values.

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# etymologia

## *Ignatzschineria* [ig-nat"shi-ner'e-ə]

A genus of aerobic, gram-negative, nonmotile rods, *Ignatzschineria* was first isolated from flies of the family Sarcophagidae (from the Greek *sarco* [“flesh”] + *phage* [“eating”]) by Erika Tóth et al. in 2001. Tóth named the genus after Austrian entomologist Ignaz Rudolph Schiner (1813–1873), who first described the fly *Wohlfahrtia magnifica*. In 2007, Tóth discovered that *Schineria* had already been used for genus of tachina flies and proposed the replacement genus name *Ignatzschineria*.

Ignaz Rudolph Schiner
Photographer unknown.
Image via Wikimedia Commons.


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# *Burkholderia pseudomallei* Infection in US Traveler Returning from Mexico, 2014

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**To the Editor:** Melioidosis is an infection with clinical manifestations ranging from skin abscess to overwhelming sepsis and death. It is caused by *Burkholderia pseudomallei*, a gram-negative, saprophytic bacillus found in soil and water. Melioidosis is highly endemic to Southeast Asia and northern Australia, and endemic to the Indian subcontinent, southern China, Hong Kong, and Taiwan (*1*).

The extent of melioidosis in the Western Hemisphere is unknown. However, new endemic foci have been identified in Puerto Rico and Brazil, and sporadic cases have been reported in other parts of the Caribbean, Central America, and South America (*2*–*5*). Melioidosis is rare in the United States; 0–5 cases are reported annually, and most cases occur in travelers returning from disease-endemic areas (*2,3*). Case clusters have been associated with extreme weather events, such as tropical storms or heavy rainfall (*5,6*). We report a case of melioidosis in a returned traveler from Los Cabos, Mexico, after Hurricane Odile.

In September 2014, a 59-year-old woman came to a hospital in Chicago, Illinois, USA, with a 4-day history of right-sided upper back and anterior chest pain, fevers, and shortness of breath. She had diabetes mellitus and well-controlled HIV infection; and had received a cadaveric renal transplant 13 months earlier. Her medications included tacrolimus, prednisone, and mycophenolate. She had traveled to Los Cabos, Mexico, 7 days before admission and was present when Hurricane Odile hit the area.

On admission to the hospital, her temperature was 38.5°C, and she had right chest wall tenderness. Her leukocyte count was $22.27 \times 10^3$ cells/mL. A computed tomography scan of the chest showed an irregular mass in the apical segment of the right upper lobe suggestive of a Pancoast tumor, with ground glass opacities and an enlarged right paratracheal lymph node.

She was given intravenous vancomycin, ceftriaxone, and levofloxacin. One of 2 admission blood cultures grew gram-negative rods after 30 h incubation, and antimicrobial drugs were changed to piperacillin/tazobactam. The isolate grew on blood, MacConkey, and chocolate agar when subcultured and was oxidase positive. It was susceptible to piperacillin/tazobactam, trimethoprim/sulfamethoxazole, and doxycycline but resistant to aminoglycosides and cephalosporins. Pending final identification of the bacterium, the patient was discharged on hospital day 5 and was given oral doxycycline for presumed bacteremic pneumonia.

The isolate was later identified as *B. pseudomallei* by using phenotype methods and PCR analysis at the Illinois Department of Public Health (Chicago, IL, USA) and confirmed as *B. pseudomallei* by the Centers for Disease Control and Prevention (Atlanta, GA, USA). Genetic analysis identified multilocus sequence type 92 and internal transcribed sequence type G, which is consistent with an isolate that originated in the Western Hemisphere (*7*).

Soon afterwards, the patient was readmitted with recurrent fevers and chest pain. Blood cultures were negative, and a computed tomography scan of the chest showed new partial cavitation of the right lung mass. Antimicrobial drugs were changed to intravenous meropenem, and immunosuppressive drugs were reduced. Oral doxycycline was added to meropenem after a third admission for recurrent fevers.

Although current international guidelines recommend a minimum of 10–14 days of intravenous therapy for melioidosis (intensive phase), relapse rates are high. A newer guideline recommends a longer intensive phase for some infections on the basis of results of an ongoing study in which longer courses were associated with lower relapse rates (*8*). Given this patient's recurrent symptoms and immunosuppression, we extended her intensive treatment phase to 6 weeks, and she showed subsequent clinical improvement.

Transition to oral eradication-phase therapy was complicated by the patient's allergy to trimethoprim/sulfamethoxazole, which is considered first-line therapy. Because treatment with oral amoxicillin/clavulanate or doxycycline has been associated with high relapse rates (*9*), we opted to give our patient combined amoxicillin/clavulanate and doxycycline to complete 6 months of antimicrobial drug therapy. She remains well 10.5 months after presentation.

Clinical diagnosis of melioidosis in nonendemic areas is challenging because signs of the disease are nonspecific and similar to those of more common diseases, such as tuberculosis. Laboratory diagnosis is also challenging. In this case, *B. pseudomallei* grew readily in culture. However, the MicroScan Walk-Away System (Beckman Coulter Inc., Brea, CA, USA) or matrix-assisted laser desorption/ionization time of flight mass spectrometry did not provide definitive species identification. For this method to be potentially useful for identification of *B. pseudomallei*, the

database used would require optimization with addition of reference spectra for the organism and its close relatives (e.g., *B. thailandensis*). *B. pseudomallei*, although different from other *Burkholderia* spp. in its pathogenicity and epidemiology, is not easily discriminated from *B. thailandensis* or *B. cepacia* complex by using phenotypic tests (*10*).

In summary, infection with *B. pseudomallei* should be considered in patients with pneumonia after travel to the Baja Peninsula in Mexico, and especially after an extreme weather event. Because of risk for transmission to laboratory workers and the potential for *B. pseudomallei* to be used for bioterrorism, clinical laboratories should perform only limited work up of suspected isolates before referring them to a public health laboratory for definitive identification.

### Acknowledgment

We used the Multi-Locus Sequence Typing website (http://www.mlst.net) at Imperial College London, developed by David Aanensen and supported by the Wellcome Trust.

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# Zika Virus Outbreak, Bahia, Brazil

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**To the Editor:** Zika virus (ZIKV) is a mosquito-borne flavivirus related to yellow fever virus, dengue virus (DENV), and West Nile virus (WNV). It is a single-stranded positive RNA virus (10,794-nt genome) that is closely related to the Spondweni virus and is transmitted by many *Aedes* spp. mosquitoes, including *Ae. africanus*, *Ae. luteocephalus*, *Ae. hensilli*, and *Ae. aegypti*. The virus was identified in rhesus monkeys during sylvatic yellow fever surveillance in the Zika Forest in Uganda in 1947 and was reported in humans in 1952 (*1*).

In 2007, an outbreak of ZIKV was reported in Yap Island, Federated States of Micronesia (*2*). ZIKV also caused a major epidemic in the French Polynesia in 2013–2014 (*3*), and New Caledonia reported imported cases from French Polynesia in 2013 and reported an outbreak in 2014 (*4*).

A new challenge has arisen in Brazil with the emergence of ZIKV and co-circulation with others arboviruses (i.e., DENV and chikungunya virus [CHIKV]). We report ZIKV infection in Brazil associated with a recent ongoing outbreak in Camaçari, Bahia, Brazil, of an illness characterized by maculopapular rash, fever, myalgias/arthralgia, and conjunctivitis.

On March 26, 2015, serum samples were obtained from 24 patients (Table) at Santa Helena Hospital in Camaçari who were given a presumptive diagnosis of an acute viral illness by emergency department physicians. These patients were given treatment for a dengue-like illness, and blood samples were obtained for complete blood counts and serologic testing by using an ELISA specific for IgG and IgM against DENV.

Serum samples were analyzed at the Federal University of Bahia by reverse transcription PCR (RT-PCR) to detect DENV, CHIKV, WNV, Mayaro virus, and ZIKV. In brief, serum samples were subjected to RNA extraction by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse transcribed by using the SuperScript II Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) and subjected to PCRs specific for DENV (*5*) CHIKV (*6*), WNV (*7*) and Mayaro virus (*8*). A positive RT-PCR for a partial region of the envelope gene with primers ZIKVENF and ZIKVENVR (positions

**Table.** Characteristics of 24 patients with positive and negative results for infection with Zika virus, Brazil, 2015

| Reverse transcription PCR result for Zika virus (no.) | Mean (SD) patient age, y | Patient sex, F/M | No. (%) | | | |
|---|---|---|---|---|---|---|
| | | | Rash | Fever | Myalgia | Headache |
| Positive (7) | 33 (15) | 6/1 | 6 (85.7) | 3 (43) | 4 (57.1) | 3 (43) |
| Negative (17) | 31 (8.5) | 12/5 | 12 (70.6) | 6 (35.3) | 9 (53) | 11 (64.7) |

1538–1558 and 1902–1883, respectively) (*9*) was considered indicative of ZIKV infection. PCR products (362 bp) were sequenced at the ACTGene Analises Moleculares, Alvorada, Rio Grande do Sul (Porto Allegre, Brazil), and sequences were deposited in GenBank under accession nos. KR816333–KR816336.

All patients were negative by RT-PCR for DENV, Mayaro virus, and WNV. Samples from 7 (29.2%) patients were positive by RT-PCR for ZIKV (369-bp fragment) and from 3 (12.5%) patients for CHIKV (305-bp fragment). There was no simultaneous detection of ZIKV and CHIKV. Most (85.7%) patients positive for ZIKV were women; they had a median age of 28 years and no history of international travel. Patients positive for ZIKV sought medical care after a 4-day (range 1–5 days) history of rash, myalgias, arthralgias, or fever. Three patients had IgG against DENV, which is consistent with a previous DENV infection, and none of the 7 ZIKV-positive patients had a positive response for DENV.

Mean laboratory findings for patients with acute ZIKV infection were a leukocyte count of 3,750 cells/mm$^3$ (range 2,790 cells/mm$^3$–6,150 cells/mm$^3$) and a platelet count of 180,000 platelets/mm$^3$ (range 151,000 platelets/mm$^3$–274,000 platelets/mm$^3$). The mean C-reactive protein level was 16.3 mg/L (range 0.9 mg/L–19.7 mg/L). Sign and symptom duration was 1–5 days, and most patients had a maculopapular rash, myalgias, fever, and headache. Arthralgia was seen less frequently.

ZIKV infections were assessed by sequencing partial ZIKV envelope gene regions of isolates. Phylogenetic analysis rooted with Spondwei virus showed that ZIKV sequences obtained belonged to the Asian lineage and showed 99% identity with a sequence from a ZIKV isolate from French Polynesia (KJ776791) (*10*).

We report ZIKV infection in Brazil in association with an ongoing outbreak of an acute maculoexantematic illness. Although the patient population samples were not randomly selected, 42% (10/24) of the patients were positive for ZIKV (n = 7) or CHIKV (n = 3) and had maculopapular rash, fever, myalgias and headache. After detection of ZIKV in Bahia, many cases have been identified in other states (http://www.promedmail.org, archive no. 20152015602.343.1158).

Cases of infection with DENV, CHIKV, and ZIKV in Brazil and elsewhere will make diagnosis based on clinical and epidemiologic grounds unreliable. These issues show the need for laboratory confirmation of these arboviral infections. More studies are needed to address the effects of these concurrent arboviruses infections in Brazil.

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# Zika Virus Transmission from French Polynesia to Brazil

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**To the Editor:** Campos et al. (*1*) reported a Zika virus (ZIKV) outbreak in Brazil in 2015. This response adds complementary data related to the propagation of this mosquitoborne disease.

To date, the largest ZIKV outbreak occurred in French Polynesia during 2013–2014. The outbreak spread to other Pacific Islands: New Caledonia, Cook Islands, Easter Island, Vanuatu, and Solomon Islands (*2*). The origin of introduction of ZIKV to French Polynesia remains unknown; introduction of ZIKV in New Caledonia was after imported cases from French Polynesia (*3*); introduction to Easter Island was suspected to have occurred among attendees of the annual Tapati festival, including those from French Polynesia (*4*). The virus was likely transmitted to New Caledonia, Cook Islands, and Easter Island when infected travelers from French Polynesia were bitten by vectors while on the islands. Frequent travel between New Caledonia and Vanuatu is likely related to the introduction of ZIKV in the latter country.

Phylogenetic studies showed that the closest strain to the one that emerged in Brazil was isolated from samples from case-patients in French Polynesia and spread among the Pacific Islands (*1*); both strains belong to the Asian lineage. It has been assumed that ZIKV was introduced to Brazil during a World Cup soccer competition in 2014 (*5*), although no ZIKV-endemic Pacific countries competed. However, in August 2014, the Va'a World Sprint Championship canoe race was held in Rio de Janeiro, Brazil. Four Pacific countries (French Polynesia, New Caledonia, Cook Islands, and Easter Island) in which ZIKV circulated during 2014 had teams engaged in this contest in several categories. These data combined with phylogenetic studies by Zanluca et al. (*5*) suggest that ZIKV introduction in Brazil may have been a consequence of this event. In areas where potential vectors are present, vigilance should be enhanced to detect imported cases of ZIKV, and laboratory capacity to confirm suspected ZIKV infections should be strengthened.

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# Schistosomiasis Screening of Travelers from Italy with Possible Exposure in Corsica, France

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**To the Editor:** Since 2014, many cases of urogenital schistosomiasis acquired in Corsica, France, have been described (*1–4*). The infections, which all occurred in persons who had bathed in the Cavu River in 2011 or 2013, represent the first cases of autochthonous *Schistosoma haematobium* infection acquired in Europe since the last reported case in Portugal in 1965 (*5*). In June 2014, France established a screening program for persons reporting exposure to the Cavu River during 2011–2013. By March 2015, a national surveillance journal had reported 110 autochthonous urogenital schistosomiasis cases in residents of France (*6*).

We describe the diagnostic work-up for and clinical management of persons from Italy who reported bathing in the Cavu River at least once during 2011–2014. All of the patients had requested screening after learning of the risk for acquiring schistosomiasis after freshwater exposure in Corsica. Exclusion criteria for the study included

**Table.** Demographic, epidemiologic, clinical, and laboratory data for 15 patients with urogenital schistosomiasis acquired after bathing in the Cavu River, Corsica, France*

| Patient age, y/sex | Year exposed | Previous symptoms | Eosinophils, cells/μL† | No. samples tested for ova | ELISA‡ | IFAT | WB§ | Infection definition |
|---|---|---|---|---|---|---|---|---|
| 12/M | 2012 | Urgency to urinate | 210 | 3 Neg | Neg: T1, T2 | ND | Neg: WB1, WB2 | Possible |
| 12/M | 2012 | None | 550 | 3 Neg | Neg: T1, T2 | ND | Neg: WB1, WB2 | Possible |
| 68/M | 2012 | Acute prostatitis | 190 | 3 Neg | Neg: T1, T2 | ND | Neg: WB1, WB2 | Possible |
| 5/M | 2011, 2012, 2013 | None | 560 | 3 Neg | Neg: T2 | ND | Neg: WB1, WB2 | Possible |
| 64/M | 1990–2013 | Macroscopic hematuria, hematospermia | 140 | 1 Neg | Pos: T1; Neg: T2 | ND | Neg: WB1, WB2 | Probable |
| 57/M | 1997, 1998, 2006–2014 | None | ND | 1 Neg | Neg: T1, T2 | ND | Neg: WB1, WB2 | Possible |
| 58/F | 1997, 1998, 2006–2014 | Macroscopic hematuria | 1,540 | 1 Neg | Pos: T1; Neg: T2 | ND | Neg: WB1, WB2 | Probable |
| 37/M | 2013 | None | 380 | 1 Neg | ND | Neg | Neg: WB1; Pos: WB2 | Confirmed |
| 54/M | 2011 | None | 110 | 1 Neg | ND | Neg | Neg: WB1; Pos: WB2 | Confirmed |
| 60/F | 2014 | None | 190 | 1 Neg | ND | Neg | Neg: WB1; Pos: WB2 | Confirmed |
| 58/M | 2011, 2012, 2013 | None | 400 | 1 Neg | ND | Neg | Neg: WB1; Pos: WB2 | Confirmed |
| 11/F | 2011, 2012, 2013 | Vaginal discharge | 500 | 3 Neg | ND | Neg | Neg: WB1; Pos: WB2 | Confirmed |
| 39/M | 1980–2013 | Urolithiasis | 40 | 3 Neg | ND | Neg | Neg: WB1, WB2 | Possible |
| 29/M | 2014 | Hematospermia | 130 | 4 Neg | ND | Neg | Neg: WB1, WB2 | Possible |
| 10/M | 2011 | None | 437 | 1 Neg | ND | Neg | Neg: WB1, WB2 | Possible |

*Only 1 patient, the 10-year-old male, had microscopic hematuria. IIFAT, indirect immunofluorescent antibody test; ND, not done; Neg, negative; Pos, positive; WB, Western blot.
†Absolute cell count.
‡T1 indicates the ELISA used in Udine and Brescia, Italy, and T2 indicates the ELISA used in Negrar, Italy.
§WB1 contained *Schistosomiasis mansoni* soluble antigens; WB2 contained *S. haematobium* plus *S. mansoni* soluble antigens.

residence in or travel to a country where schistosomiasis is endemic.

At least 3 months after their last exposure to the Cavu River, each participant had a filtered terminal urine sample and a serum sample tested for schistosomiasis. Different commercial tests were used, depending on local availability: 3 different ELISAs and an indirect immunofluorescent antibody test (IIFAT). All serum samples were tested in parallel in a laboratory in Florence, Italy, by using 2 Western blots (WBs): a Schistosoma WB IgG kit containing antigens from adult *S. mansoni* worms and a second kit containing *S. mansoni* and *S. haematobium* antigens from a crude adult extract (LDBio Diagnostics, Lyon, France).

Confirmed urogenital schistosomiasis was defined by confirmation of *S. haematobium* eggs in urine by microscopy, positive WB result, or both. Probable urogenital schistosomiasis was defined by positive serologic test results. Possible urogenital schistosomiasis was defined by signs or symptoms suggestive of schistosomiasis (i.e., urogenital symptoms), eosinophilia (>0.4 × $10^9$ cells/L of blood), or both (*7*). All participants who met the case definition received 1 oral dose of praziquantel (40 mg/kg).

Forty-three persons were consecutively enrolled during January 2014–January 2015; of these, 15 (34%) had confirmed (6 patients), probable (2 patients), or possible (7 patients) urogenital schistosomiasis (Table). Of these 15 patients, 7 (47%) reported repeat visits to Cavu River over a period of at least 2 years. The mean eosinophil count was 295 (range 40–1,540) cells/μL of blood; 6 (40%) patients had eosinophilia. Genitourinary symptoms were reported by 7 (47%) patients, and blood was detected by dipstick in the urine of 1 patient. Schistosoma eggs were not found in any urine samples.

Schistosomiasis screening has been suggested for persons with exposure to the Cavu River (*6*); however, clinical history and clinical evaluation alone and eosinophilia, have low sensitivity for the diagnosis of urogenital schistosomiasis (*7*,*8*). Asymptomatic infection has been reported in 25%–36% of persons with travel-associated schistosomiasis, and eosinophilia was present in 50% of the patients (*7*,*8*). In screenings in France, only 27% of schistosomiasis-positive patients reported genitourinary symptoms (*6*).

For the diagnosis of urogenital schistosomiasis, serologic testing is more sensitive than detection of eggs in urine, particularly in mild infections (*7*–*9*). Many asymptomatic family members of the index case-patients who acquired infection in Corsica tested positive only by serologic testing (*1*–*4*). However, commercial serologic tests for schistosomiasis have low sensitivity (*9*). Kinkel et al. (*9*) showed that sensitivity of an IIFAT and 3 ELISAs for *S. haematobium* ranged from 21.4% to 71.4%. In the Corsica outbreak, serologic testing may be even less sensitive because of the hybrid

nature of the schistosoma (*S. haematobium*/*S. bovis*) (*6*). In our study, only 2 patients had positive ELISA results. Combinations of >2 serologic tests can markedly increase testing sensitivity to almost 78.6% (*9*).

Sulahian et al. (*10*) found that a WB containing *S. mansoni* antigens had 89.5% sensitivity and 100% specificity for *S. mansoni*. In our study, no patients with urogenital schistosomiasis tested positive by WB containing *S. mansoni* antigens, but 6 patients tested positive by WB containing *S. haematobium* antigens.

In mild infections, the absence of schistosoma antibodies cannot exclude a diagnosis of urogenital schistosomiasis (*7*). Therefore, we provided treatment to patients with possible urogenital schistosomiasis; our decision to treat these patients considered the tolerability of praziquantel and the possible severe genitourinary complications of untreated infections (e.g., bladder carcinoma, infertility).

Our findings suggest that a sensitive screening strategy for urogenital schistosomiasis consists of a patient's travel history (exposure in multiple years), clinical history (any new genitourinary complaints after freshwater exposure), eosinophil count, and serologic testing. Because of the failure of commercial ELISA and IIFAT methods, we emphasize that a WB containing *S. haematobium* antigen should also be used for screening.

Of note, a confirmed urogenital schistosomiasis case acquired after a single exposure in 2014 was never reported (*1*–*4*,*6*). The risk for delayed diagnosis of this insidious, neglected disease, which has recently reappeared in Europe, must be reduced. To accomplish this, information regarding the risk for schistosomiasis after freshwater exposure in Corsica must be disseminated to physicians worldwide.

**Acknowledgment**

We thank colleagues for useful suggestions regarding the manuscript and for providing help in its preparation.

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# Anthropology of Infectious Disease

**By Merrill Springer.** 320 pages. Walnut Creek, CA, USA: Left Coast Press, 2014. Hardback: $94, ISBN: 978-1-62958-044-2; paperback, $32.95: ISBN: 978-1-62958-044-9; e-book, $32.95, ISBN: 978-1-62958-044-3.

Merrill Singer's Anthropology of Infectious Disease argues that pathogens are intertwined with human social worlds. Through a variety of case studies drawn from around the world, from HIV to malaria and from Lyme disease to tuberculosis, the book emphasizes a biosocial or biocultural approach to the understanding of infectious disease. In contrast to a strictly biomedical framework, the core argument of the book is that infectious diseases cannot be understood through biology alone but rather must be considered within the context of the cultural and social worlds they inhabit. The book emphasizes the interactions between biologic, political, economic, sociocultural, and ecologic factors and how these factors affect the emergence, prevention, treatment, distribution, cultural experiences, and global impact of pathogens.

The first 2 chapters of the book provide the framework for the rest of the text. The first chapter begins with a history of the anthropology of infectious disease, starting from the post–World War II period to the present day. This chapter continues by making a strong case for the inclusion of anthropologic frameworks in our understanding of microbes and their impact because ethnography and other anthropologic tools link every day on-the-ground experiences to broader social structures and global processes. Following this foundation in social science, the book's second chapter provides a more biologically based introduction to microbes, emphasizing human–pathogen interaction and evolution.

Using theoretical approaches ranging from ecosocial theory, medical ecologic theory, phenomenologic and meaning-centered approaches, and critical medical anthropology, Singer explores a wide array of topics throughout the rest of the book. Drawing from medical ecologic theory, the next 3 chapters explore connections between humans, the environment, and other organisms. These linkages are explored through the lens of multispecies infections and zoonotic diseases, the effect of environmental devastation on patterns of disease, and the evolutionary arms race between humans and emerging drug-resistant pathogens. Moving on to the role of social factors in the differential burden of disease in various populations, the last sections of the book explore social experiences of suffering, highlighting the interaction of infectious with noninfectious disease, the relationship between inequality and emerging infectious disease, and the effects of politics and global structural forces on future pathogenic challenges.

Throughout the text, Singer presents a broad array of examples. The volume of case studies drawn upon, although useful in their analytic breadth, could be overwhelming for the novice reader. Thus, the book could benefit from an emphasis on a few core case studies.

Overall, however, Anthropology of Infectious Disease is written clearly and compellingly and would make an important addition to a course in public health, medical anthropology, or even microbiology. Although the book could be assigned as a whole, each chapter can stand alone, making it a versatile and practical teaching tool for students at multiple levels. In addition, the book contains a comprehensive glossary and discussion questions at the end of each chapter, making it even more useful in educational settings. Because of its accessible style and clear elucidation of theory, this book is also appropriate for practitioners in medicine or public health and infectious disease who would like to familiarize themselves with social science approaches in the field.

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**John James Audubon (1785–1851). *Cow-pen Bird*, Plate XCIX (99)** Engraved from watercolor. 19.5 × 12.25 in. / 49.53 × 31.12 cm. Image from Archives Service Center, University of Pittsburgh, Pittsburgh, PA, USA.

# Don't Lay Your Eggs All in One Basket: Brood Parasitism as a Survival Strategy

**Byron Breedlove and Paul M. Arguin**

This month's cover image,[1] Plate 99 from *Birds of America* (printed in stages during 1827–1838) by American ornithologist, naturalist, and painter John James Audubon (1785–1851), shows a pair of oft-vilified brown-headed cowbirds. This painting appears in the book as one of 435 life-sized watercolors that were reproduced from Audubon's hand-engraved plates. In this painting, he portrays the birds frozen in act of foraging, a technique he honed from observing birds where they lived. Audubon's own words best describe the work: "Male with the head and neck sooty-brown, the body black, glossed with green, the fore part of the back with blue. Female considerably smaller, greyish-brown, the lower parts lighter."

The genus of this bird, *Molothrus ater*—as well as the bronze-headed variant *Molothrus aeneus*—comes from Molothrus, the Greek work meaning vagabond or parasite. Although there are other bird species that prey on the parental skills of their feathered neighbors by laying eggs in their nests, only birds of those two species practice obligate parasitism in North America, placing them among approximately one percent of avian species worldwide.

Before cowbirds—which are also variously known as cow-pen birds, cow buntings, or buffalo birds—followed cattle, they tracked bison herds across the Great Plains, where they were sustained by the copious insects. Naturalists have ventured that cowbirds adapted to this nomadic existence by becoming brood parasites and depositing their eggs in nests built and incubated by birds of other species. Incubation for birds is analogous to pregnancy for mammals, providing warmth, protection, and food to the developing embryo. This process can involve establishing and defending a territory, nest building, and collecting food

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[1]This image from John James Audubon's *Birds of America* is a reproduction obtained from the University of Pittsburgh, which has in its collections one of the 120 complete sets known to exist.

not for individual consumption, but for feeding the newly hatched chicks.

David Attenborough in his book *The Life of Birds* succinctly describes the advantages of such an adaptation: "Brood parasitism relieves the parasitic parent from the investment of rearing young or building nests for the young, enabling them to spend more time on other activities such as foraging and producing offspring. The risk of egg loss is mitigated, as eggs are distributed amongst a number of different hosts." During a laying season, brood parasites often produce many more eggs than their parasitized hosts. By distributing these eggs among many different nests, they also spread the risk for nest predation, increasing the chances that some of their offspring will survive.

Skillful at stealth and stalking, brown-headed cowbirds parasitize birds of about 200 other species throughout North America; researchers have observed that birds of 144 of those species have raised cowbird offspring. Raising cowbird hatchlings among a brood extracts a toll on host species, despite countermeasures ranging from rejecting or destroying the intruder's eggs or chicks to abandoning the parasitized nests. Cowbird eggs usually hatch a day ahead of the host's own eggs, and cowbird nestlings usually are larger and mature faster than the host's young, enabling them to consume more of the food their foster parents bring to the nest. Despite the fact that 97% of cowbird eggs and nestlings do not survive to adulthood, brood parasitism by cowbirds has pushed birds of some host species to the status of "endangered" and has probably hurt populations of birds of some other host species.

Brood parasitism, of course, is but one variant on the nonsymbiotic relationship between species, both animals and plants, in which one benefits at the expense of the other. Medical parasites are often larger and more complex than the average bacterium or virus, yet they depend on other organisms to complete their life cycle. They live on or in other species, using them as a food source, for shelter, or for some other essential aspect of their daily life. The actual number of parasitic organisms on earth is likely inestimable and certainly debatable; some researchers have postulated that 40% of the species in any location are parasitic.

Three main classes of parasites—protozoa, helminths, and ectoparasites—cause numerous diseases afflicting millions of humans. Of all parasitic diseases, malaria causes the most deaths globally, and other parasitic diseases such as lymphatic filariasis, onchocerciasis, and schistosomiasis afflict millions of people in the tropics and subtropics. Parasitic diseases can be imported into or in some cases transmitted within industrialized countries. In the United States, for example, tickborne transmission of babesiosis and foodborne outbreaks of cyclosporiasis and anisakiasis are just some of the parasitic infections of public health concern. Enhanced surveillance, treatment, and prevention of parasitic infections remain global health priorities.

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# EMERGING INFECTIOUS DISEASES®

## Upcoming Issue

- Ebola in West Africa—CDC's Role in Epidemic Detection, Control, and Prevention
- Uncommon *Candida* Species Fungemia in Cancer Patients, Houston, Texas, USA
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- Carbapenem-Resistant *Enterobacteriaceae* in Children, United States, 1999–2012
- Contact Tracing Activities during the Ebola Virus Disease Epidemic in Kindia and Faranah, Guinea, 2014
- Role of Maternal Antibodies in Infants with Severe Diseases Related to Human Parechovirus Type 3
- Molecular Epidemiology of Hospital Outbreak of Middle East Respiratory Syndrome, Riyadh, Saudi Arabia, 2014
- Association of Higher MERS-CoV Virus Load with Severe Disease and Death, Saudi Arabia, 2014
- Climatic Influences on *Cryptoccoccus gattii* Populations, Vancouver Island, Canada
- Coccidioidomycosis among Construction Workers, California, USA, 2014
- No Geographic Correlation between Lyme Disease and Death due to 4 Neurodegenerative Disorders, United States, 2001–2010
- USA300 Methicillin-Resistant *Staphylococcus aureus*, United States, 2000–2013
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- Invasive Pneumococcal Disease 3 Years after Introduction of 10-Valent Pneumococcal Conjugate Vaccine, the Netherlands
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- Mortality Risk Factors for Middle East Respiratory Syndrome Outbreak, South Korea, 2015
- Pneumococcal Infection among Children before Introduction of 13-Valent Pneumococcal Conjugate Vaccine, Cambodia
- RmtC and RmtF 16S rRNA Methyltransferase in NDM-1–Producing *Pseudomonas aeruginosa*

**Complete list of articles in the November issue at http://www.cdc.gov/eid/upcoming.htm**

## Upcoming Infectious Disease Activities

**October 25–29, 2015**
**ASTMH**
**American Society of Tropical Medicine and Hygiene**
**64th Annual Meeting**
**Philadelphia, PA, USA**
**info@astmh.org**
**https://www.astmh.org/Home1.htm**

**October 31–November 4, 2015**
**APHA**
**143rd Annual Meeting & Expo**
**Chicago, IL, USA**
**http://apha.org/events-and-meetings/annual**

**December 6–9, 2015**
**2015 National HIV Prevention Conference**
**Atlanta, GA**
**http://www.cdc.gov/nhpc/index.html**

**February 8–10, 2016**
**ASM Biodefense and Emerging Diseases Research Meeting**
**Arlington, VA, USA**
**biodefense@asmusa.org**
**http://www.asmbiodefense.org/**

**March 2–5, 2016**
**ISID**
**17th International Congress on Infectious Diseases**
**Hyderabad, India**
**http://www.isid.org/icid**

## Announcements

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## Article Title

## Invasive Disease Caused by Nontypeable *Haemophilus influenzae*

## CME Questions

**1. You are consulting for a large health maintenance organization regarding planning strategies to deal with invasive nontypeable *Haemophilus influenzae*. According to the review by Langereis and colleagues, which of the following statements about recent evidence regarding the emergence of invasive nontypeable *H. influenzae* is correct?**

A. The number of recorded cases doubled in the last 2 decades
B. Nontypeable *H. influenzae* invasion was detected mainly in adolescents
C. Nontypeable *H. influenzae* invasion develops as a pneumonia or bacteremia without apparent focus of infection
D. Emergence of nontypeable *H. influenzae* as a cause of invasive disease is restricted to The Netherlands

**2. According to the review by Langereis and colleagues, which of the following statements about mechanisms that may explain the increasing prevalence of invasive nontypeable *H. influenzae* is correct?**

A. Use of the Hib vaccine and polysaccharide conjugate vaccine do not affect *H. influenzae* strain replacement
B. The emergence of nontypeable *H. influenzae* as a cause for invasive disease is solely the result of an increase in the number of disease cases
C. Increased bacterial virulence is not a proposed mechanism contributing to invasive nontypeable *H. influenzae*
D. The epidemiology of nontypeable *H. influenzae* has changed dramatically in the last 20 years, now affecting mostly elderly persons, perhaps because of increased comorbidity compromising immunologic status

**3. According to the review by Langereis and colleagues, which of the following statements about potential strategies to implement effective prevention of invasive nontypeable *H. influenzae* would most likely be correct?**

A. Development of vaccines against nontypeable *H. influenzae* is not likely to play a significant role
B. Development of an effective vaccine for risk groups demands clarification of the factors contributing to the emergence of invasive nontypeable *H. influenzae* disease, such as age and comorbidity
C. Reducing nontypeable *H. influenzae* colonization in children is unlikely to help prevent invasive disease
D. Broad vaccination strategies in the general public are unlikely to help prevent invasive nontypeable *H. influenzae* in those at greatest risk

## 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 75% passing score) and earn continuing medical education (CME) credit, please go to http://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 "Register" link on the right 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/about-ama/awards/ama-physicians-recognition-award.page. 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

## Cluster of *Cryptococcus neoformans* Infections in Intensive Care Unit, Arkansas, USA, 2013

## CME Questions

**1. You are seeing a 70-year-old woman with diabetes and severe chronic kidney disease referred to the emergency department for pneumonia unresponsive to outpatient treatment with antibiotics. You consider atypical organisms that may cause pneumonia, including *Cryptococcus neoformans*. Which of the following statements regarding the epidemiology and presentation of infection with *C. neoformans* is most accurate?**

**A. *C. neoformans* is found only in North America**
B. The initial infection with *C. neoformans* is usually asymptomatic
C. Primary infection with *C. neoformans* promotes the highest rates of morbidity and mortality
D. Bloodstream infections account for the majority of cases of reactivation of *C. neoformans*

**2. What should you consider regarding the clinical presentations and outcomes of patients with *C. neoformans* infection in the current study?**

A. Most patients had bloodstream infection
B. Most patients had a history of HIV infection
C. Nearly all patients had pneumonia
D. The mortality rate was approximately 30%

**3. Which of the following variables was found to contribute most significantly to the outbreak of *C. neoformans* in the current study?**

A. Attendance at the same church
B. Employment at the same poultry processing plant
C. Exposure to a healthcare worker who was a carrier of *C. neoformans*
D. None of the above

**4. Which of the following treatment variables was most associated with infection with *C. neoformans* in the current study cohort?**

A. Treatment with multiple broad-spectrum antibiotics
B. Treatment with hemodialysis
C. Failure to promote pulmonary hygiene
D. Short-term treatment with corticosteroids

## 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** 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.

## Summary of Authors' Instructions

**Author's Instructions.** For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

**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. Set the document to show continuous line numbers. 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 (not to exceed 15) 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.