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History of Mosquitoborne Diseases in the United States and Implications for New Pathogens

Max J. Moreno-Madriñán, Michael Turell

The introduction and spread of West Nile virus and the recent introduction of chikungunya and Zika viruses into the Americas have raised concern about the potential for various tropical pathogens to become established in North America. A historical analysis of yellow fever and malaria incidences in the United States suggests that it is not merely a temperate climate that keeps these pathogens from becoming established. Instead, socioeconomic changes are the most likely explanation for why these pathogens essentially disappeared from the United States yet remain a problem in tropical areas. In contrast to these anthropogenic pathogens that require humans in their transmission cycle, zoonotic pathogens are only slightly affected by socioeconomic factors, which is why West Nile virus became established in North America. In light of increasing globalization, we need to be concerned about the introduction of pathogens such as Rift Valley fever, Japanese encephalitis, and Venezuelan equine encephalitis viruses.

The recent explosive outbreaks of disease throughout the Americas caused by the introduction of Zika and chikungunya viruses has raised several questions, including whether these or similar disease-causing pathogens could spread into countries located at temperate latitudes, particularly into the continental United States. The conventional perception is that diseases caused by mosquito-transmitted pathogens are mostly associated with tropical areas. Indeed, such areas include the ranges of temperature and other climatic conditions that are ideal for the vectors of these pathogens. Moreover, because such diseases are clearly more prevalent in these areas, we might easily assume that the association between the tropics and mosquito-transmitted pathogens indicates that temperate regions are at less risk for these diseases because of their cooler climates. However, this expectation of safety should not be taken for granted. In fact, cases of disease caused by mosquito-transmitted pathogens such as West Nile virus (WNV) occur readily in North America, and several encephalitides occasionally occur in the United States. In addition, history reveals that yellow fever and malaria were once very common in the United States and resulted in millions of cases. More than 100,000 deaths occurred in the United States in the 18th and 19th centuries from yellow fever alone (Figure), in areas considered as temperate or cold according to the Köppen-Geiger climate classification scheme.

Historical Aspects

The Aedes aegypti mosquito and 2 of its transmitted viruses (yellow fever and dengue viruses) and the plasmodia parasites of malaria (Plasmodium vivax and P. falciparum) are believed to have arrived to the Americas during the 17th century by ship during the slave trade. During the 17th century (even during a cold period known as the Little Ice Age) until the 19th century, summertime malaria was present in much of the eastern United States, including northern areas of the country. Numerous outbreaks of malaria occurred as far north as Massachusetts, with documented outbreaks occurring during 1793–1799 and in 1806, 1810, 1820, 1828, and 1836; nearly 2,000 cases were reported during an outbreak in 1880 alone. In the subtropical southern states along the Mississippi Valley during the 18th and 19th centuries, malaria spread quickly, especially during the American Revolutionary War and Civil War. Malaria is reported to have caused ≈1,300,000 cases of illness and ≈10,000 deaths among soldiers during the 4 years of the Civil War. Although mosquito transmission of disease-causing organisms was not proved until 1889, the coincidental use of window and door screens might have contributed to the lowering prevalence by the beginning of the 1880s.

Efforts to control mosquitoes by draining mosquito larval habitat sites have been undertaken since 1930 by the US government. However, such efforts might have been improperly managed, and the depopulation of the rural South likely was the main factor leading to the substantial reduction in malaria by the early 1940s. In 1947, the National Malaria Eradication Program, a cooperation between the state and local health agencies

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of 13 southeastern states and the Communicable Disease Center of the US Public Health Service, commenced the operations that presumably resulted in the elimination of malaria from the United States (14). The application of DDT and drainage of mosquito larval habitats were the main control methods used. By the early 1950s, after 3 years with no indigenous cases (based on eradication criteria stated by the National Malaria Society at the time), malaria was considered to be no longer endemic in any given area of the continental United States (14). Although 63 outbreaks of locally transmitted mosquito-borne malaria were reported in the United States during 1957–2003, these outbreaks consisted of only 156 cases (15) and were caused by mosquitoes infected after biting persons that had acquired the pathogen in other countries (16). Still, concern about malaria exists because the 3 mosquito species that transmitted malaria in the United States before its eradication, Anopheles quadrivittatus, An. freeborni, and An. pseudopunctipennis, along with other anopheline species, are still present in the country (16). Thus, rather than the mere control of the vector, the introduction of screening, air conditioning, television, and other enhancements (i.e., improved socioeconomic conditions), along with improved early diagnosis and access to treatment, might also have helped eliminate human reservoirs and eradicate malaria from the United States.

Yellow fever epidemics were common in northeastern US cities as far north as Boston, Massachusetts; Portsmouth, New Hampshire; and Providence, Rhode Island, during the 18th century and the beginning of the 19th century (4,17). The large epidemic of yellow fever in Philadelphia, Pennsylvania, in the summer of 1793 (resulting in ≈5,000 deaths) was a contributing factor in the decision to move the nation’s capital to the city of Washington (18), and epidemics in 1798 in Philadelphia and New York, New York, resulted in 3,500 and 2,080 deaths, respectively (4). After 1822, yellow fever epidemics in the United States were generally restricted to more southern cities (4). In addition, major epidemics occurred in New Orleans, Louisiana, in 1853 (≈9,000 deaths) (4) and Savannah, Georgia, in 1876 (1,066 deaths) (4). In the great epidemic of 1878, 16,000–20,000 deaths from yellow fever occurred along the Mississippi River, from the Gulf of Mexico to Memphis, Tennessee, and St. Louis, Missouri (4). Overall, at least 100,000 deaths were attributed to yellow fever in the United States during 1693–1905 (4). Within a few years after the discovery in 1901 that yellow fever was transmitted by mosquito, the last locally acquired cases of yellow fever in the United States were reported in 1905 in Pearlington, Mississippi (46 deaths), and New Orleans (437 deaths) (4,11). It is important to note that open cisterns were common in homes of the Mississippi Gulf Coast from the 18th century through the early 20th century and that ≈5,000 deaths from yellow
fever occurred in Mississippi roughly within the same period (11). Important aspects in commonly characterizing these yellow fever epidemics were the tendency for them to occur during summer and fall and in port cities with active trade with the Caribbean Islands (Figure). Today, these aspects still represent a concern but now are not just limited to port cities as our increasingly global society experiences increasing numbers of travelers flying from tropical areas (19), especially during the summer season.

On the basis of identification of *Ae. aegypti* mosquitoes as the primary vector of yellow fever by the Walter Reed Commission in 1901 and the earlier discovery by Ronald Ross that the parasite that causes malaria is transmitted by mosquito (20), the first actions taken by US companies that took over the construction of the Panama Canal in Panama was the eradication of mosquitoes, mainly by draining or oiling of larval rearing sites and fumigation with pyrethrum (21). In 1947, the Pan American Sanitary Bureau, later renamed the Pan American Health Organization, implemented a program that lasted until 1970 to eradicate *Ae. aegypti* mosquitoes and consequently dengue (7). The mosquito was declared eradicated from 18 continental countries in the Americas in 1962 (22). The apparent success of the elimination program, along with environmental concerns about the use of pesticides such as DDT, were among the reasons to end it. However, since 1971, *Ae. aegypti* mosquitoes have started regaining their previous geographic distribution, including a notable increase occurring after 2000 (7). The failure to eradicate this vector in the Americas was a major factor leading to the reemergence of dengue (22) and very likely to the recent appearance of chikungunya and Zika viruses. Dengue outbreaks have continued to occur in southern Texas and southern Florida as recently as 2011 (23,24). However, these outbreaks have been more associated with the high proportion of persons traveling from dengue-endemic countries than to climatic or socioeconomic conditions.

**Socioeconomic Changes and Anthroponotic Pathogens**

As we have mentioned, the mosquito vectors capable of transmitting malaria, yellow fever, and dengue have been present throughout much of the United States since the 1600s (4,7,11). What has clearly changed in the United States from the 18th and 19th centuries to the present is the availability of potable water, sanitation, and social lifestyles. These developments have essentially eliminated the need to store water in indoor containers and reduced contact with mosquitoes. After World War II, and particularly during the 1950s, a boom in the US economy increased the standard of living and aided the widespread use of television and air conditioning. In addition, the use of screened terraces and windows increased. These commodities influenced persons to spend a longer time indoors or in screened areas (thus decreasing outdoor exposure to mosquito bites) and made indoor environments less accessible to outside mosquitoes. Because changes in living conditions in the United States reduced the opportunity for contact between mosquitoes and humans, these changes substantially affected the transmission of those pathogens for which humans are the primary amplifying host. Such pathogens fall into the group of anthroponoses (i.e., those in which humans are the principal vertebrate host). Thus, diseases such as malaria, yellow fever, and dengue have all but disappeared (4,7,9,14–16), and the viruses that cause chikungunya and Zika, which arrived into the United States after living conditions had improved, have not become established in the continental United States despite the occurrence of ≈10,000 imported cases of these infections since 2014 (25,26).

The impact of changing cultural and socioeconomic conditions on the prevalence of anthroponotic diseases over time has also been observed when the cultural and socioeconomic conditions change across geographic areas. Contemporary observations of dengue prevalence in contiguous cities at the United States–Mexico border indicate lower dengue prevalence and higher socioeconomic conditions on the US side (27,28). The lower rates of dengue, chikungunya, and Zika virus infection reported in the United States compared with Latin America countries coincide with higher socioeconomic conditions in the United States (29). Although malaria and yellow fever are transmitted by different mosquitoes and the larval habitats and biting behaviors of these mosquitoes are different, both diseases are similar in that humans are essentially the only amplifying vertebrate host. Therefore, continued transmission would require considerable mosquito–human contact, and either a reduction in the number of mosquitoes present or their ability to bite humans would be necessary to reduce the potential for continued transmission.

**Potential for the Introduction of Zoonotic Viruses**

This association between socioeconomic status and prevalence of diseases caused by mosquito-transmitted pathogens applies more to anthroponotic than to zoonotic pathogens (i.e., those pathogens in which animals other than humans play a major role in the transmission cycle). These zoonotic viruses cause diseases such as WNV infection, St. Louis encephalitis, eastern equine encephalitis, western equine encephalitis, and La Crosse encephalitis. These viruses are maintained in natural transmission cycles involving various mosquito and bird or rodent species and therefore are not usually greatly affected by improved housing for humans. Because humans are not involved in the transmission cycle of these viruses, the viruses persist in the United States, whereas anthroponotic pathogens (e.g., malaria and dengue and yellow fever viruses), which involve only
humans as vertebrate amplifying hosts, have been essentially eliminated (4,7,8,14–16). In contrast, zoonotic viruses, such as eastern equine encephalitis and La Crosse viruses, continue to be a cause of disease (30,31), and the introduction of WNV in 1999 (32) illustrates the potential for an exotic virus to become established in North America. Since its introduction in 1999, WNV has spread throughout the continental United States as well as to southern Canada and most of Central and South America (33,34). In the continental United States, ≈40,000 cases and ≈2,000 deaths have been attributed to WNV infection (2). Thus, unlike the anthropogenic viruses chikungunya and Zika, which failed to become established in the continental United States despite the introduction of ≈10,000 imported cases (25,26), exotic zoonotic viruses could very likely be introduced and become established, with potentially devastating consequences.

**Recent History of the Introduction of Novel Viruses**

Like yellow fever and dengue viruses, chikungunya and Zika viruses are also anthropogenic viruses transmitted by *Ae. aegypti* mosquitoes. These viruses were recently introduced into the Americas, where each has caused massive outbreaks in South and Central America as well as in the Caribbean, resulting in ≈2 million infections with chikungunya virus (35) and ≈750,000 infections with Zika virus (36). These outbreaks have led to the introduction of numerous imported cases into the continental United States, where ≈3,500 imported cases of chikungunya virus infection (25) and ≈5,000 imported cases of Zika virus infection (26) have been reported to the Centers for Disease Control and Prevention (CDC). In addition to the *Ae. aegypti* mosquito, the *Ae. albopictus* mosquito has been implicated as a potential vector of Zika (37,38) and chikungunya (39,40) viruses. The first locally acquired chikungunya case in the continental United States was reported in Florida in July 2014 (41). As of December 31, 2016, CDC has reported 3,869 chikungunya cases in the United States, of which 13, all in southern Florida, have been identified as locally transmitted (25). Zika virus is of particular concern because of its potential to cause Guillaum-Barré syndrome and congenital abnormalities such as microcephaly (42,43) and because it can be transmitted not only through mosquito bites but also from mother to child, by blood transfusion, and through sexual contact (44). Indeed, the first case identified in the United States (in 2008) was attributed to sexual transmission (45). As of July 12, 2017, CDC has reported a total of 5,381 cases of Zika virus infection in the continental United States, including 49 cases acquired through sexual transmission or routes other than mosquito transmission and 224 cases assumed to be transmitted locally by mosquito bite (26). Local mosquito transmission was first reported in July 2016 in southern Florida (46), and genetic studies indicate that 4–40 separate introductions of the virus contributed to the outbreaks (47). In addition, similar to dengue and chikungunya, cases of locally transmitted Zika virus have been identified in southern Texas (48). Most of these introductions originated from the Caribbean, as indicated by genetic and surveillance analysis and supported by the high influx of travelers from this high-incidence region (47). As we previously mentioned, the active trade between the Caribbean and the United States played a key role in influencing the abundance of yellow fever outbreaks in the United States during 1693–1905 (4). This history demonstrates not only the potential threat for the arrival of new pathogens from tropical origins but, in general, the risk of globalization for the introduction of new pathogens as a whole and the importance of paying attention to the public health of developing countries as a way to ensure the safety of local public health everywhere.

**Conclusions**

Although anthropogenic pathogens such as yellow fever virus and malaria were once prevalent in the United States (including the northern states), their prevalence occurred when socioeconomic conditions were poor. However, when these conditions improved, such diseases virtually disappeared. Since 2014, despite the introduction of ≈10,000 imported cases of Zika or chikungunya virus infections, relatively few local cases have been reported, and all of them occurred in areas where *Ae. aegypti* mosquitoes were present (i.e., neither of these 2 viruses has managed to become established in the continental United States). On the other hand, as illustrated by WNV, a zoonotic virus has the potential to be introduced and become established. Zika and chikungunya viruses failed to become established because they are anthropogenic viruses, whereas WNV was able to become established because it is a zoonotic virus (some other zoonotic viruses such as Japanese encephalitis and Rift Valley fever viruses show similar potential). Improvement in standards of living inhibits anthropogenic but not zoonotic viruses. Given that the decreased exposure of humans to mosquitoes in the United States is primarily driven by changes in socioeconomic conditions, it is important to note that these very conditions could be threatened by massive natural disasters or any other similarly disruptive event. Consequently, appropriate disaster preparedness plans need to be in place to address this potential threat.

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**Emerging Infectious Diseases**

A large number of imported cases of Zika virus infection and the potential for transmission by *Aedes albopictus* mosquitoes prompted the New York City Department of Health and Mental Hygiene to conduct sentinel, enhanced passive, and syndromic surveillance for locally acquired mosquitoborne Zika virus infections in New York City, NY, USA, during June–October 2016. Suspected case-patients were those >5 years of age without a travel

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1Working group members who participated in this study are listed at the end of this article.
 Zika virus, an arbovirus of the genus *Flavivirus*, has spread rapidly across Latin America and the Caribbean region after an epidemic was identified in Brazil in early 2015 (1,2). Although it is usually clinically mild or asymptomatic, Zika virus infection during pregnancy can cause microcephaly and other severe brain, eye, and ear defects in the fetus (3,4). Among adults, Zika virus has also been linked to neurologic disorders, including Guillain-Barré syndrome (5).

The primary vector of the Zika virus epidemic, the mosquito *Aedes aegypti*, has not been found in New York City (NYC), NY, USA; however, the less-efficient mosquito vector *Ae. albopictus* is present throughout the city (6–11). Historically, NYC has not had local transmission of either dengue or chikungunya viruses, which are also spread by *Ae. aegypti* and *Ae. albopictus* mosquitoes.

NYC is a destination for a large population of travelers, as well as being the home of ≈1.8 million persons from the Caribbean region and Latin America, who might travel back and forth to Zika-affected areas (12–14). As of June 17, 2016, there were 182 confirmed cases of Zika virus infection in NYC, one of the highest case burdens in the United States (15).

The Centers for Disease Control and Prevention (CDC) interim response plan for Zika recommends enhanced surveillance in areas with *Ae. aegypti* mosquitoes (16). The risk for local mosquitoborne transmission in NYC was thought to be less than in jurisdictions with *Ae. aegypti* mosquitoes. However, local transmission was still a concern given the high number of travel-associated cases, the nascent knowledge about Zika transmission, and the potential need to rapidly implement local control measures should mosquito-borne transmission be demonstrated (17). In response, the NYC Department of Health and Mental Hygiene (DOHMH) enhanced both human surveillance and mosquito control efforts during 2016 (15). This report describes the establishment and outcomes of sentinel, enhanced citywide passive, and emergency department (ED) syndromic surveillance systems to identify potential human cases of local mosquito-borne transmission of Zika virus.

### Methods

#### Sentinel Surveillance

DOHMH identified the first travel-associated Zika cases in NYC in January 2016 (15); the number of cases peaked in June 2016 (NYC DOHMH, unpub. data). Sentinel surveillance for local transmission was initiated in June 2016. Sentinel surveillance relies on detection of disease in facilities likelier to see cases, can require fewer public health resources than population-based surveillance, and can provide more detailed data than passive reporting (18). We selected facilities throughout the city for the sentinel surveillance network on the basis of locations in neighborhoods with high counts of reported cases of travel-associated Zika virus infection, historically elevated counts of travel-associated dengue or chikungunya diseases from Zika-affected countries, an environmental habitat conducive to *Ae. albopictus* mosquito breeding, or areas with large adult *Ae. albopictus* mosquito populations.

Participating sentinel clinical sites received patient screening criteria, reporting instructions, sterile urine collection tubes, educational posters, and, in some cases, mosquito repellents to distribute to persons planning to travel to Zika-affected areas. Sentinel sites used a paper DOHMH reporting form to capture clinical and demographic information on suspected cases.

One designated healthcare staff member at each site received weekly DOHMH update emails and was responsible for disseminating the sentinel case definition and any relevant updates to other providers onsite. These providers were of varying medical specialties, including internal medicine, emergency medicine, infectious disease, family medicine, and pediatrics. For assistance, providers could also contact the regular DOHMH 24-hour on-call physician or a direct sentinel call number active during the surveillance period.

The initial definition of a suspected case-patient from sentinel surveillance was any patient >5 years of age who reported no travel to a Zika-affected area within the previous 4 weeks and showed ≥3 of the 4 major Zika signs/symptoms: fever, rash, arthralgia, or conjunctivitis. Because children frequently have fever and rash, patients ≤5 years of age were excluded because of low specificity of the symptom-based case definition in this population (16). An exception was made for a 1-year-old patient who had all 4 signs/symptoms and reported no travel to a Zika-affected country.

#### Enhanced Passive Surveillance

Enhanced passive surveillance is an amplification of standard passive surveillance in which public health agencies send notifications to healthcare providers and facilities to remain alert for suspected cases of a particular disease or
condition (19). In July 2016, local transmission of Zika virus in Florida prompted the expansion of sentinel surveillance to enhanced passive surveillance starting in August 2016 (20). DOHMH used the Health Alert Network, an email-based public health alert system, to encourage all physicians to seek Zika virus testing for eligible patients. The final case definition for sentinel and passive sites was any patient who met all of the following criteria: ≥5 years of age; ≥3 of 4 signs/symptoms (arthralgia, fever, conjunctivitis, and rash); no history of travel to a Zika-affected area in the previous 4 weeks; no history of sex with a person who traveled to a Zika-affected area in the previous 4 weeks; and urine specimen collected within 14 days after illness onset.

**Routine Case Investigation**

Following DOHMH protocol, epidemiologists interviewed patients who had laboratory evidence of Zika virus infection (or their guardians). During these interviews, investigators asked patients whether they had any nonsexual household contacts who developed Zika-like signs/symptoms and whether the contact had traveled to a Zika-affected area. Any reports of symptomatic, nonsexual household contacts without travel were assessed for risk and referred to testing if appropriate.

**Laboratory Testing**

Patients who met the sentinel case definition were asked to provide ≥3 mL of urine in sterile tubes. According to CDC guidelines, urine must have been obtained within 14 days after illness onset to be eligible for testing (21). The urine samples were stored at 4°C until they could be transported on ice to the NYC DOHMH Public Health Laboratory (PHL) for testing. DOHMH arranged for transportation of specimens via a courier service. Pregnant patients were requested to submit not only urine specimens but also serum specimens. All sentinel specimens were prioritized (within 48 hours) for Zika virus RNA testing by a real-time reverse transcription PCR (rRT-PCR) assay at PHL (22). We assessed timeliness of PHL testing using the diagnosis time, defined as the number of hours between specimen collection at the healthcare facility and rRT-PCR result availability, as well as testing time, defined as the number of hours between specimen arrival at PHL and rRT-PCR result availability.

**Syndromic Surveillance**

Syndromic surveillance uses electronic health-related data in near–real time to assess the health of a community with the goal of early identification of disease clusters or cases (23). The NYC DOHMH syndromic surveillance system uses daily visit data from all NYC EDs. For visits by patients ≥5 years of age, chief complaint text and International Classification of Diseases (ICD) version 9 and 10 diagnosis codes were used to create a fever syndrome for spatiotemporal analysis and a Zika-like illness line list for case finding.

Patients with fever visits were identified as those with chief complaint terms fever, febrile, or pyrexia or with ICD version 9 diagnosis code 780.6 or ICD version 10 diagnosis code R50. Fever was chosen as a surrogate marker for potential locally acquired Zika virus disease because fever was reported by ≈80% of symptomatic NYC patients with travel-associated Zika virus disease at the time of sign/symptom selection; is uncommon among persons >5 years of age during mosquito season in NYC; and was found to be more specific than rash, conjunctivitis, or arthralgia, with less variety in patient chief complaint.

Each day, we applied the prospective space-time permutation scan statistic using SaTScan version 9.4 invoked in batch mode through SAS version 9.4 to identify spatiotemporal clusters of fever syndrome by hospital ZIP code and by patient residential ZIP code (24). The input file was for 90 days, the maximum spatial cluster size was 20% of observed visits, and the maximum temporal cluster size was 14 days. Initially, we defined a signal as a cluster with a recurrence interval ≥100 days, but to limit false signaling, on June 18, 2016, we redefined a signal as a cluster with a recurrence interval ≥365 days. A recurrence interval represents the number of days of daily surveillance required for the expected number of clusters at least as unusual as the observed cluster to be equal to 1 by chance (25). We defined unique clusters as clusters with no spatial overlap with ZIP codes or hospitals identified in the prior day’s most likely cluster. We overlaid clusters on a map of areas identified using a statistical model as being at high risk of Zika virus importation in any given week. We evaluated spatiotemporal clusters qualitatively, taking into consideration the recurrence interval, whether hospitals included in the cluster recently transitioned to patient tracking and data transfer using Health Level Seven (HL7) international reporting standards (http://www.hl7.org), cluster size relative to the estimated <200 m range of the *Ae. albopictus* mosquito, and any geographic overlap with areas at high risk for Zika virus importation (26).

In addition, patients with Zika-like illness were identified through ED visits with mention of any of the following scenarios: chief complaint including at least 3 signs/symptoms among rash, fever, joint pain, or conjunctivitis; chief complaint including the term Zika; diagnosis of Guillain-Barré syndrome; or diagnosis of arboviral infection. Initially, DOHMH staff members reviewed all ED visits for Zika-like illness. As the volume of visits increased through June and July, a case finding pilot was conducted to
determine whether syndromic surveillance could identify nontravel patients for testing and, if so, establish rules for when follow-up investigation was necessary. During the pilot period, July 31–August 4, 2016, DOHMH surveillance analysts contacted hospital staff to collect information on travel history, diagnosis, and any Zika testing of patients identified as having Zika-like illness. Because this work was conducted in the course of routine surveillance and public health practice, institutional review board approval was not required.

Results

Sentinel and Enhanced Passive Surveillance
The NYC DOHMH sentinel surveillance system for locally acquired Zika virus infection was operational during June 27–September 30, 2016, and consisted of 24 NYC hospitals and community health centers. Sentinel sites were located in all 5 boroughs: 7 in the Bronx, 6 in Queens, 5 in Brooklyn, 3 in Manhattan, and 3 in Staten Island. Enhanced passive surveillance was instituted on August 2, 2016.

A total of 15 patients met the suspected case definition; of these, 8 (53%) were reported from 5 sentinel sites and 7 (47%) from 6 nonsentinel sites (Table), including 4 hospitals and 2 outpatient centers. The most common location of residence was the Bronx (n = 5), followed by Manhattan (n = 3) and Queens (n = 3). All patients from the Bronx sought care at sentinel sites. The median patient age was 35 years (interquartile range [IQR] 20–49 years). Nine (60%) patients were female. Two patients were pregnant at the time of testing. No household contacts of cases were referred for Zika testing during routine case investigation.

The median diagnosis time for all submissions was 46.3 hours (IQR 27–98 hours), and the median testing time was 6.2 hours (IQR 6–26 hours). No specimens had Zika virus RNA detected by rRT-PCR.

Fever Syndrome ED Visits
We performed automated spatiotemporal cluster detection analyses for fever syndrome daily on visits from all 53 NYC EDs during June 1–October 31, 2016. During this period, there were 40,073 visits for fever syndrome, with a daily median of 262 visits (IQR 247–277 visits). We detected 17 unique spatiotemporal fever syndrome clusters. Upon investigation, we discarded 2 clusters with low recurrence intervals, because these clusters would not have signaled after applying the final signaling threshold. We examined 14 other clusters that we identified as artifacts resulting from 17 hospitals transitioning during the analysis period to using HL7 international reporting standards. The transition resulted in longer fields for the chief complaint, and thus more opportunity to include a fever keyword, causing localized increased syndrome capture compared with the baseline. The remaining cluster did not overlap with areas classified as high risk for Zika importation and had a radius of 11.1 km, inconsistent with the estimated <200 m range of *Ae. albopictus* mosquitoes (26).

Zika-Like Illness ED Visits
We identified 308 ED visits for Zika-like illness during June 1–October 31, 2016 (Figure); daily median was 2 visits (IQR 1–3 visits). During the case finding pilot, we identified 19 Zika-like illness visits at 17 hospitals. For 6 visits, DOHMH surveillance analysts could not reach hospital staff for follow-up after 3 attempts or medical records were unavailable. Of 13 (68%) visits with completed follow-up, travel to Zika-affected countries was confirmed for 85% of patients. The remaining 15% of patients with completed follow-up and no travel were found to have diagnoses inconsistent with Zika virus infection. Follow-up determined that all visits with mention of the term Zika were for patients with travel history and had been appropriately assessed for infection risk and testing by the ED physician.

Table. Demographic and clinical characteristics of persons meeting case definition for suspicion of local transmission of Zika virus at sentinel and nonsentinel passive surveillance sites in New York City, June 27–September 30, 2016*

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total tested for Zika virus</th>
<th>Nonsentinel site</th>
<th>Sentinel site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number tested</td>
<td>15 (100)</td>
<td>7 (47)</td>
<td>8 (53)</td>
</tr>
<tr>
<td>Median age (interquartile range)</td>
<td>35 (20–49)</td>
<td>29 (9–80)</td>
<td>45 (21–54)</td>
</tr>
<tr>
<td>Borough</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bronx</td>
<td>5 (33)</td>
<td>1 (14)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Brooklyn</td>
<td>2 (13)</td>
<td>1 (14)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>Manhattan</td>
<td>3 (20)</td>
<td>2 (29)</td>
<td>1 (13)</td>
</tr>
<tr>
<td>Queens</td>
<td>3 (20)</td>
<td>3 (43)</td>
<td>0</td>
</tr>
<tr>
<td>Staten Island</td>
<td>2 (13)</td>
<td>0</td>
<td>2 (25)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>9 (60)</td>
<td>6 (86)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>M</td>
<td>6 (40)</td>
<td>1 (14)</td>
<td>5 (62)</td>
</tr>
<tr>
<td>Pregnant at time of report</td>
<td>2 (13)</td>
<td>2 (29)</td>
<td>0</td>
</tr>
<tr>
<td>Signs and symptoms</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthralgia</td>
<td>12 (80)</td>
<td>4 (57)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Conjunctivitis</td>
<td>10 (67)</td>
<td>7 (100)</td>
<td>3 (38)</td>
</tr>
<tr>
<td>Fever</td>
<td>13 (86.7)</td>
<td>5 (71.4)</td>
<td>8 (100)</td>
</tr>
<tr>
<td>Rash</td>
<td>11 (73.3)</td>
<td>4 (57.1)</td>
<td>7 (87.5)</td>
</tr>
</tbody>
</table>

*Values are no. (%) except as indicated. Signs and symptoms are not mutually exclusive.
Surveillance for Mosquitoborne Zika Virus

Based on the case finding pilot, we established that follow-up case investigation was not necessary when the visit text had one of the following: mention of recent travel to a country or territory with local Zika virus transmission, an alternate diagnosis listed, or a chief complaint containing “Zika.” Following this protocol, during August 5–October 31, 2016, a total of 12 of 163 Zika-like illness visits qualified for additional follow-up: 8 were found to have traveled to Zika-affected countries, but this information was not noted in the original chief complaint or travel history data fields; 1 patient had already been tested for Zika virus; 1 patient was ruled out because medical records noted allergic reaction with no fever; 1 patient was admitted to labor and delivery from the ED and the ED medical record was inaccessible to the hospital staff contact; and 1 patient could not be matched to hospital records on the basis of information available in the syndromic record.

Discussion

During the peak period of travel-associated Zika cases and the mosquito season in NYC in 2016, DOHMH used case investigation, sentinel, enhanced passive, and syndromic surveillance to monitor for the presence of local mosquitoborne Zika virus transmission. None of the systems detected any local mosquitoborne transmission.

During the sentinel surveillance period, healthcare providers identified 15 NYC residents without recent travel history or sexual exposure who met the sentinel case definition for Zika virus; all tested negative for Zika virus. Only 21% of sentinel sites reported any suspected cases, which might indicate a lack of engagement of some sites, inappropriate selection of site locations, or a true lack of patients who met the criteria. Future iterations of a sentinel surveillance program should include an evaluation component, such as the deployment of a mock patient with locally acquired Zika virus infection or a review of medical records, to evaluate provider awareness and effectiveness of the sentinel sites.

Although DOHMH targeted efforts to identify suspected locally acquired cases at presumed higher-risk sentinel sites, 47% of patients tested for Zika virus were seen at nonsentinel sites. We believe this might have been because Zika virus infection is a reportable disease in New York and healthcare facilities were already sending specimens from suspected travel-associated Zika virus cases to PHL for testing (27). Given that baseline knowledge regarding Zika may have been heightened, the additional contact with sentinel sites may not have appreciably improved reporting.

Enhanced passive citywide surveillance has the advantages of including all NYC residents and relying on the preexisting reportable disease system. Potential downsides to this open system are the incorrect application of screening criteria and subsequent overburdening of laboratories with test requests. In response to the high volume of travel-associated Zika virus testing through PHL, by March 2016 DOHMH had implemented a system to screen test requests for appropriateness (15). This same system was used to screen test requests of suspected local transmission, mitigating the potential for incorrect application of screening criteria.

CDC guidelines for early detection of possible local transmission require timely testing of patients (16). For both sentinel and passive surveillance, the use of urine specimens for Zika virus testing was less invasive for patients and allowed health centers without laboratory capabilities...
to submit specimens, because serum testing requires specimen processing with a centrifuge shortly after collection. DOHMH also arranged for the transport of specimens for testing to PHL, reducing provider burden. The median time between specimen collection and result availability was 46 hours, indicating that PHL was able to efficiently process and report suspected cases of local mosquitoborne transmission. The caveat to testing urine only is that Zika virus RNA may persist longer in serum than in urine (28).

During June–October 2016, no concerning spatiotemporal clusters of fever were identified through syndromic surveillance. Because available spatial elements of the data were limited to hospital location and patient residential ZIP code, spatiotemporal clusters associated with patient workplace were not detectable. In addition, although the initial choice of fever syndrome alone was appropriate for NYC at the start of the case finding, by the end of the study period, rash was identified as the most prevalent sign or symptom of Zika virus infection. This finding is in line with a CDC study of travel-associated cases during January–March 2016 that found that rash was the most common sign or symptom reported (98%), followed by fever (82%) and arthralgia (66%) (29). Requiring ≥2 signs/symptoms, rather than ≥3, would have been less sensitive for cluster detection because of patients who experience or report only 1 relevant sign/symptom and given the restrictions in the length of the chief complaint text provided by some hospitals. Although the incidence of other febrile illnesses during the summer in NYC is low, particularly compared with illnesses with rash, evaluation on the basis of fever syndrome may not be as appropriate in tropical settings where high incidence of febrile illnesses might circulate concurrently with Zika virus. Rash should be evaluated for future syndromic surveillance, with consideration for potential background levels of rash during the summer.

During August–October 2016, a manageable number (12 of 163) of ED visits for Zika-like illness met protocol criteria for DOHMH staff to follow up with hospital infection control practitioners regarding patient travel history. Follow-up activities would be challenging to sustain with a higher volume of cases. The pilot study highlighted the importance of collecting travel history data and led DOHMH to request during the study period that clinical facilities include an additional travel history field in the daily ED data transmission to improve capture of patient travel. Continued collaboration between DOHMH and EDs to improve travel data will facilitate surveillance for Zika virus as well as for other travel-associated diseases.

A limitation of the syndromic surveillance system was the incomplete transition of all hospitals to HL7 international reporting standards, resulting in differences in the average number of terms in the ED chief complaint per hospital and precluding use of a more specific, multiple-symptom definition for cluster detection. For the Zika-like illness linelist, requiring at least 3 signs/symptoms of Zika virus infection biased case detection toward hospitals that provide more detailed chief complaints, which might not have corresponded to areas where the risk of local mosquitoborne transmission was highest in NYC.

The major limitation of all surveillance for local transmission of Zika virus is that 80% of Zika virus infections are asymptomatic; therefore, any surveillance system reliant on the presence of symptoms will underestimate the true incidence of infection (4,30). Thus, the surveillance systems used by DOHMH might have missed capturing smaller, local outbreaks of mosquitoborne Zika virus. In particular, spatiotemporal cluster detection requires many symptomatic persons seeking care within a specified interval. Therefore, it is unlikely that syndromic surveillance would be sufficiently sensitive to detect a small cluster of locally acquired infections.

Despite the stated limitations, our experience suggests that enhanced passive surveillance, with frequent outreach to providers in communities with large numbers of travel-associated human cases or habitats conducive to potentially competent mosquito vectors, was an efficient and manageable method to monitor for locally transmitted mosquitoborne Zika virus infection in NYC. Given that NYC does not have *Ae. aegypti* mosquitoes, the multipronged surveillance approach taken by NYC DOHMH during the first year of the epidemic was considered a robust plan to detect local mosquitoborne transmission of Zika virus. The likelihood that another vectorborne infection will emerge somewhere in the world is high, and surveillance is a critical tool for the detection and evaluation of control measures (31,32). Our experience offers a possible surveillance model for other jurisdictions concerned about the possibility of local mosquitoborne Zika virus or other arboviral transmission.
Alfonso Chan, Joseph P. Addabbo Family Health Center—Arverne and Joseph P. Addabbo Family Health Center—Far Rockaway; William Mariano, NYC Health + Hospitals/Kings County; Erica Cardona, Medisys Health Network Center—East New York; Bill Killip, Staten Island University Hospital; Cesar Del Rosario, NYC Health + Hospitals/Woodhull; Robert Chin, NYC Health + Hospitals/Woodhull; Stacy Nunberg, Bronx–Lennon Hospital Center; Michael Irvin, The Institute for Family Health–Mount Hope Family Practice; Andy Choi, Northwell Health–GoHealth Urgent Care–Forest Hills and Northwell Health–GoHealth Urgent Care–Lefrak City

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References
SYNOPSIS


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- An Operational Framework for Insecticide Resistance Management Planning
- *Rickettsia parkeri* Rickettsiosis, Arizona, USA
- *Plasmodium falciparum* In Vitro Resistance to Monodesethylamodiaquine, Dakar, Senegal, 2014
- Astrovirus MLB2, a New Gastroenteric Virus Associated with Meningitis and Disseminated Infection
- Spectrum of Viral Pathogens in Blood of Malaria-Free III Travelers Returning to Canada
- Expanded Geographic Distribution and Clinical Characteristics of *Ehrlichia ewingii* Infections, United States
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Two Cases of Israeli Spotted Fever with Purpura Fulminans, Sharon District, Israel

Regev Cohen, Frida Babushkin, Maurice Shapiro, Martina Uda, Yafit Atiya-Nasagi, Dar Klein, Talya Finn

We report a series of 5 case-patients who had Israeli spotted fever, of whom 2 had purpura fulminans and died. Four case-patients were given a diagnosis on the basis of PCR of skin biopsy specimens 3–4 days after treatment with doxycycline; 1 case-patient was given a diagnosis on the basis of seroconversion. Rickettsia spp. from the 2 case-patients who died were sequenced and identified as Rickettsia.
conorii subsp. israelensis. Purpura fulminans has been described in association with *R. rickettsii* and *R. indica*, but rarely with *R. conorii* subsp. *israelensis*.

*Rickettsia conorii* is the etiologic agent of Mediterranean spotted fever (MSF), which is considered one of the most severe and life-threatening rickettsial infections. Among the 4 strains of *R. conorii* (*R. conorii* subsp. *conorii*, *R. conorii* subsp. caspia, *R. conorii* subsp. *indica*, and *R. conorii* subsp. *israelensis*), *R. conorii* subsp. *israelensis*, which causes Israeli spotted fever (ISF), is believed to be the most virulent strain and shows a case-fatality rate of up to 32.3% in hospitalized patients (1). *R. conorii* is endemic to Israel (2). However, in recent years, *Rickettsia* spp. other than *R. conorii* have been identified and reported from Israel, including *R. africae*, *R. massiliae*, and *R. sibirica* (3,4).

Within the *R. conorii* group, there are clinical and virulence differences. For example, infections with *R. conorii* subsp. *israelensis* have higher case-fatality rates than infections with the *R. conorii* Malish strain (29% vs. 13%), and *R. conorii* subsp. *israelensis* is rarely associated with an eschar at the site of a tick bite (5,6). ISF has also been reported from other Mediterranean countries, including Portugal (7), Italy and Sicily (6), and Tunisia (8). *R. conorii* subsp. *conorii* that cause MSF are also endemic to Europe, Asia, and Africa.

ISF usually manifests as fever with a maculopapular rash, usually involving the palms of the hands and the soles of the feet. Patients who die from ISF typically have multiorgan failure, acute renal or hepatic failure, and acute encephalitis; all are attributed to the affinity of the *Rickettsia* spp. to endothelial cells with resultant vasculitis. The typical maculopapular rash might transform to become hemorrhagic with discrete purpurial lesions, but true manifestations of ISF purpura fulminans is not frequently reported in Israel or from other countries.

Because serologic cross-reactivity occurs across the spotted fever group (SFG) rickettsiae (9) and the primary means of diagnosis is through serum antibody assays, accurate distinction between different subspecies requires identification of the actual infecting bacterium. This cross-reactivity, overlap in geographic distributions, and the different clinical severities highlight the need to better differentiate between these rickettsial species. A correct diagnosis is critical for predicting the pathologic complications that would arise because of infection (10).

We report a case series of 5 patients with ISF from the same geographic area (Sharon District in Israel) (Tables 1, 2). Four of these case-patients were detected during April–May 2017. Two case-patients had purpura fulminans and both died. These cases were a part of a national outbreak of SFG rickettsiosis.

**Case-Patient 1**

A 75-year-old woman with a history of hypertension, a tourist from Ukraine who had been staying with her daughter in Netanya for the previous 3 months, came to an emergency department in October 2016 with 6 days of fever, weakness, and anorexia. She had been exposed to 3 dogs at the house of her daughter but had no recollection of tick exposure. At admission, she was coherent but in a state of septic shock; she was hypotensive and had a disseminated, fern-leaf pattern, purpurial rash over her body, including the face (Figure 1). Laboratory findings included leukocytosis, severe thrombocytopenia (23,000 platelets/µL), hypotremia (sodium level 126 mmol/L), acute renal failure, increased levels of liver enzymes and creatine kinase (CK), and disseminated intravascular coagulation (Table 1).

She was admitted to the intensive care unit (ICU) and given broad-spectrum antimicrobial drug therapy, including intravenous doxycycline. During the next 2 days, multiorgan failure, severe jaundice, liver failure, and acute lung injury developed, and the rash became bullous with clear serous fluid (Figure 2). A skin biopsy specimen from a hemorrhagic lesion was obtained and tested by PCR. The result was positive for *R. conorii* subsp. *israelensis*. Serologic analysis for rickettsia at the time of admission (day 9 after disease onset) showed no reactivity, but another sample obtained 10 days later was positive (IgG titer 1:800) for SFG rickettsiae.

**Table 1. Laboratory findings at hospitalization for 5 case-patients with Israeli spotted fever, Sharon District, Israel**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Reference range</th>
<th>Case-patient 1</th>
<th>Case-patient 2</th>
<th>Case-patient 3</th>
<th>Case-patient 4</th>
<th>Case-patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes, cells/µL</td>
<td>4,000–11,000</td>
<td>10,700</td>
<td>7,500</td>
<td>5,000</td>
<td>7,700</td>
<td>8,600</td>
</tr>
<tr>
<td>PMNs, %</td>
<td>40–75</td>
<td>87</td>
<td>86</td>
<td>81</td>
<td>85</td>
<td>91</td>
</tr>
<tr>
<td>Platelets/µL</td>
<td>150,000–400,000</td>
<td>37,000</td>
<td>73,000</td>
<td>91,000</td>
<td>75,000</td>
<td>139,000</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>136–145</td>
<td>126</td>
<td>123</td>
<td>130</td>
<td>133</td>
<td>127</td>
</tr>
<tr>
<td>Total bilirubin, mg/dL</td>
<td>0.3–1.4</td>
<td>2.5</td>
<td>0.4</td>
<td>0.9</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>AST, IU/L</td>
<td>0–32</td>
<td>106</td>
<td>184</td>
<td>167</td>
<td>65</td>
<td>69</td>
</tr>
<tr>
<td>ALT, IU/L</td>
<td>0–33</td>
<td>51</td>
<td>154</td>
<td>139</td>
<td>68</td>
<td>66</td>
</tr>
<tr>
<td>GGT, IU/L</td>
<td>0–40</td>
<td>50</td>
<td>103</td>
<td>62</td>
<td>26</td>
<td>106</td>
</tr>
<tr>
<td>LDH, IU/L</td>
<td>240–480</td>
<td>1044</td>
<td>875</td>
<td>1195</td>
<td>466</td>
<td>536</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.5–0.9</td>
<td>1.7</td>
<td>1.4</td>
<td>1.0</td>
<td>1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Creatine kinase, IU/L</td>
<td>39–190</td>
<td>298</td>
<td>1933</td>
<td>152</td>
<td>159</td>
<td>100</td>
</tr>
<tr>
<td>C-reactive protein, mg/L</td>
<td>0–5</td>
<td>122</td>
<td>350</td>
<td>164</td>
<td>89</td>
<td>240</td>
</tr>
</tbody>
</table>

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, γ-glutamyl transferase; LDH, lactate dehydrogenase; PMN, polymorphonuclear leukocytes.*
The skin lesions became dusky, necrosis appeared at the extremities (Figure 3), and there was extensive desquamation. The patient died of candidemia after 14 days in ICU.

Case-Patient 2

A 51-year-old woman with diabetes, who was mentally retarded and lived in a nursing home was hospitalized in an internal medicine ward on May 2017 with fever of no obvious source. She had no known exposure to dogs or ticks at the nursing home. The case-patient was hypotensive at admission, and laboratory results included a standard leukocyte count, thrombocytopenia (73,000 platelets/µL), hyponatremia, acute renal failure, increased levels of CK (1,933 IU/L) and liver enzymes, and a C-reactive protein level of 350 mg/L (Table 1). She was treated empirically with ceftriaxone.

Serologic analysis on day 6 of the fever showed an IgM titer >1:100 and a borderline IgG titer of 1:100 for rickettsia.

Despite treatment with doxycycline, multiorgan failure developed and the patient died of *Pseudomonas aeruginosa* bacteremia related to central line infection 3 weeks after admission.

Case-Patients 3–5

Three other case-patients were admitted to Laniado Hospital (Netanya, Israel) during April–May 2017 for fever and a maculopapular rash involving the palms that appeared on the third or fourth day after fever onset. Two patients had exposure to dogs, but none reported exposure to ticks. One patient had alcoholism, but he had a benign illness course. The diagnosis for 2 patients was made by using PCR for skin biopsy specimens, and the diagnosis for the third patient was made by using seroconversion. Skin biopsy PCR results were positive despite 3–4 days of doxycycline treatment.

Methods

Serologic Analysis

Serologic diagnosis was made by using indirect immunofluorescence assays. Serum samples were obtained from 5
patients and tested for antibodies against R. conorii and R. typhi as described (11).

DNA Extraction and PCR Detection
We extracted DNA from skin biopsy or whole blood specimens from 4 patients by using the QIAamp Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer’s instructions. We tested samples for SFG rickettsiae by using nested PCR to amplify a fragment of the 17-kDa protein antigen gene as described (12). For further identification, we performed an additional PCR by using primers derived from consensus sequences of the rickettsial outer membrane protein A gene (213F 5′-AATCAATATTGGAGCGGTAA-3′ and 667R 5′-ATTTGCAATACAGTATAAGTGAC-3′). We analyzed sequences by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). This fragment was identical to that of the Israeli tick typhus rickettsial outer membrane protein A gene (GenBank accession no. U83441.1).

Discussion
SFG rickettsial disease is endemic to Israel and might be related to deaths of infected patients. However, manifestation of ISF as primary purpura fulminans has been described only rarely. Thus, we report patients with rickettsial disease and purpura fulminans.

Among various species of SFG rickettsiae, the types known to be associated with patient deaths are R. rickettsii (agent of Rocky Mountain spotted fever); R. conorii of the subtypes conorii, israelensis, and indica; and rarely R. australis (13). Although patient deaths are associated with vasculitis and multiorgan failure, purpura fulminans as part of the clinical presentation is a feature seen only for a small subset of the species, namely R. rickettsii, R. australis (14), and probably R. conorii subsp. indica. R. conorii subsp. indica was reported in several case series from the Indian subcontinent as a cause of fern-leaf pattern purpura, although the specific rickettsial subtype was infrequently identified in these studies (15–20).

Purpura fulminans is not a typical feature of ISF in reviews and in case series from Israel and other countries to which R. conorii subsp. israelensis is endemic (5,6,21). Although there are reports of deaths of patients in Israel who had a clinical picture suspected initially to be meningococcemia, fern-leaf purpura, as for 2 of our case-patients, was not part of the clinical descriptions (22). We have found only 1 case report of R. conorii subsp. israelensis infection and purpura fulminans (23). We report 2 additional cases.

The type of severe skin damage in the 2 case-patients we report is not the natural history of fatal spotted fever cases because it has not been described in other patients dying of ISF. The severe and confluent skin lesions undoubtedly contributed to bloodstream infections, leading to death.
in both cases. Because the differential diagnosis of purpura fulminans in Israel usually includes microorganisms other than rickettsiae, this deadly, unreported, unique, and potentially misleading manifestation of ISF must be recognized and treated in a timely fashion.

The clinical presentation of case-patients in this case series was typical of ISF and compatible with other case series of ISF (Table 2) (5,24). The reasons why 2 of these patients died and the other 3 patients had relatively mild disease is not entirely clear. Both case-patients who died were older; had hypotension, purpura fulminans, multiorgan failure, and acute renal failure; and showed increased levels of CK. Delay in doxycycline administration was evident for case-patient 1 (day 6 after illness onset) because she was hospitalized at a later time. A delay in doxycycline administration during medical observation occurred for case-patient 2, although treatment was given on day 5 after disease onset, which was similar to the timing for the 3 case-patients who survived.

We sequenced rickettsiae isolated from the 2 case-patients who died; both were positive for R. conorii subsp. israelensis. However, we did not perform sequencing for isolates from the other 2 case-patients who were positive by PCR for the SFG rickettsiae. These findings suggest that disease severity might be related to the specific subtype, and not to host factors, but further research is required. Alcoholism has been reported a risk factor for fatal disease (3), but the patient in this case series who had alcoholism had a benign course of illness.

With regards to epidemiologic, clinical, and laboratory findings, as expected, none of our patients had an eschar or known exposure to ticks. One patient had diarrhea, but 2 patients had constipation. All 5 case-patients had rash and laboratory abnormalities typical of ISF (Table 1), as described (24).

As previously reported (6,24), serologic analysis was not useful in the diagnosis of ISF because results were nonreactive for all case-patients early in the disease course and positive for 2 case-patients tested during the convalescent period (19 days after disease onset). In contrast, whole blood and skin biopsy specimen PCRs were helpful in obtaining a diagnosis. PCR results for skin biopsy specimens were positive for 4 of the 5 case-patients even though these PCRs were performed for 4 patients 3–4 days after doxycycline therapy was begun. The 1 case-patient with a negative PCR result for a skin biopsy specimen also had no inflammatory changes by histologic analysis, Thus, the negative PCR result for this patient probably represented a sampling error and not a false-negative result.

PCR for whole blood was performed for 2 patients: case-patient 2 showed a positive result after 3 days of doxycycline therapy, and case-patient 5 showed a negative result after 5 days of doxycycline therapy. Reduced sensitivity of whole blood PCR after beginning antimicrobial drug therapy has been reported (24). For the 4 case-patients with positive PCR results for skin biopsy specimens, the pathology report did not indicate a diagnosis of rickettsiosis, describing either intravascular coagulation and no vasculitis for the 2 patients who died and perivascular minimal lymphocytic infiltration not consistent with rickettsiosis for the other 2 patients.

Our cluster was a part of an outbreak throughout Israel during the spring and summer of 2017. This outbreak included deaths of 2 healthy young persons. One of these persons was a 15-year-old boy from the Sharon District. During July 2017, the Israeli Ministry of Health reported on an increased incidence of spotted fever cases (11 cases during January–May 2017); the yearly average is 6 cases (25).

In summary, during the spring and summer of 2017, there was an increased incidence of spotted fever in Israel, causing the deaths of 4 patients. Three of these patients acquired the disease in the Sharon District, and 2 were seen in our facility. Two of the case-patients who died were confirmed by sequencing to be infected with R. conorii subsp. israelensis; both of these patients had purpura fulminans and multiorgan failure. Purpura fulminans should increase the suspicion of ISF, along with other better-described pathogens, such as Neisseria meningitidis, and diagnostic and therapeutic measures must be taken urgently.

PCR of skin biopsy specimens is useful in providing a rapid and accurate diagnosis, even if performed 3–4 days after beginning of treatment, and PCR of whole blood might also show positive results during antimicrobial drug treatment early in the disease course. Histopathologic results for skin lesions might be inaccurate and cause a delay in diagnosis or an erroneous diagnosis. Features of R. conorii subsp. israelensis should be further explored primarily by performing genetic sequencing of all isolates from cases of spotted fever diagnosed in Israel to distinguish it from other spotted fever rickettsiae or R. conorii subsp. conorii that might cause a less aggressive disease.

About the Author
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References

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To determine trends, mortality rates, and costs of antimicrobial resistance in invasive bacterial infections in hospitalized children, we analyzed data from Angkor Hospital for Children, Siem Reap, Cambodia, for 2007–2016. A total of 39,050 cultures yielded 1,341 target pathogens. Resistance rates were high; 82% each of Escherichia coli and Klebsiella pneumoniae isolates were multidrug resistant. Hospital-acquired isolates were more often resistant than community-acquired isolates; resistance trends over time were heterogeneous. K. pneumoniae isolates from neonates were more likely than those from nonneonates to be resistant to ampicillin–gentamicin and third-generation cephalosporins. In patients with community-acquired gram-negative bacteremia, third-generation cephalosporin resistance was associated with increased mortality rates, increased intensive care unit admissions, and 2.26-fold increased healthcare costs among survivors. High antimicrobial resistance in this setting is a threat to human life and the economy. In similar low-resource settings, our methods could be reproduced as a robust surveillance model for antimicrobial resistance.

Worldwide, invasive bacterial infections are a leading cause of childhood deaths, mostly in low- and middle-income countries (1). Management of such infections is threatened by the rising prevalence of antimicrobial resistance (AMR), particularly among neonates (2). However, data on AMR in invasive bacterial infections in children from low- and middle-income countries are scarce (3–6).

To combat the global threat of AMR, improved surveillance to detect emerging and long-term resistance trends is vital (7). Several global initiatives, such as the Fleming Fund, have been recently established to improve laboratory capacity in low- and middle-income countries (7,8), and the World Health Organization (WHO) Global Antimicrobial Resistance Surveillance System (GLASS) (9) has targeted 6 invasive pathogens for routine antimicrobial resistance surveillance: Escherichia coli, Klebsiella pneumoniae, Acinetobacter baumannii, Salmonella spp., Staphylococcus aureus, and Streptococcus pneumoniae. Monitoring resistance in these pathogens is particularly important for invasive bacterial infections in children in low- and middle-income countries, where most treatment is empirically prescribed and must be based on reliable contemporaneous resistance data to be effective.

Recent systematic reviews of AMR in invasive bacterial infections in children highlight the paucity of data available and do not report temporal resistance trends (5,6). In addition, although recent studies indicate excess deaths caused by AMR in low- and middle-income countries (10), there is limited evidence describing the economic and mortality burden of resistance at the patient level, particularly among children.

We analyzed 10 years of continuous AMR surveillance data for invasive bacterial infections in children from a sentinel surveillance site in Cambodia and describe resistance trends over time, by age group, and by site of acquisition (community or hospital). To evaluate the excess deaths and cost burden associated with third-generation cephalosporin resistance in community-acquired gram-negative bacteremia in hospitalized children, we analyzed patient-level data.
Methods

Study Design and Sample Selection
Angkor Hospital for Children is an ≈100-bed nongovernmental hospital in Siem Reap, Cambodia. Of children admitted, around two thirds reside in Siem Reap Province (11), where the incidence of poverty exceeds 50% (12); 93% are admitted from the community and 7% are transferred from another hospital (P. Turner, unpub data). Because this hospital has no maternity/obstetric ward, all children are born outside the hospital. Blood cultures are routinely taken from febrile (axillary temperature ≥37.5°C) hospitalized patients, according to clinical algorithms, at no patient cost.

We reviewed hospital microbiology data for 2007–2016 and extracted AMR data for selected blood culture and cerebrospinal fluid (CSF) culture isolates. Target organisms consisted of the 6 GLASS blood culture priority pathogens (9), Neisseria meningitidis (a vaccine-preventable pathogen), and non-GLASS pathogens for which ≥30 organisms were isolated over the study period. We included in the study the first isolate of a given organism per patient per 14-day infection episode, except for Salmonella spp., for which we included only the first isolate per patient to avoid double counting potential relapses. Clinical data were extracted from hospital patient records. The study was approved by the Angkor Hospital for Children Institutional Review Board (AHC-IRB, 0185-17) and the Oxford Tropical Research Ethics Committee (OxTREC, 508-17).

Procedures and AMR Reporting
We processed blood and CSF culture specimens as described elsewhere (11,13). Antimicrobial susceptibility testing was undertaken by disk diffusion and Etest MIC, according to Clinical and Laboratory Standards Institute guidelines (14) (online Technical Appendix Methods and Table 1, https://wwwnc.cdc.gov/EID/article/24/5/17-1830-Techapp1.pdf). Resistance proportions are reported as number of resistant isolates/number of isolates tested.

Outcome Analyses
We included in patient outcome analyses community-acquired monomicrobial Enterobacteriaceae (excluding Salmonellae) and A. baumannii bacteremia. These pathogens represent common causes of sepsis in children worldwide where third-generation cephalosporins would be a first-line/empiric treatment. We obtained clinical and costing data from hospital records and calculated cost per patient as admission cost plus antimicrobial costs.

Statistical Analyses
We treated isolates from specimens taken within 48 hours of admission as community-acquired infections and after 48 hours as hospital-acquired infections. However, Salmonella enterica serotypes Typhi and Paratyphi and Burkholderia pseudomallei isolates were always considered community-acquired infections. To ensure sufficient data per period, we grouped isolates into 2-year blocks. We assessed associations between resistance and year of isolation, patient age group, and site of acquisition (community vs. hospital) by univariable and multivariable logistic regression. Multivariable models included all variables. According to assessment of model fit by calculation of Akaike information criterion and plotting of observed versus predicted data, we considered time (year of isolation) a factor unless otherwise stated.

For the outcome analyses, we used univariable analysis to compare variables by third-generation cephalosporin resistance status and patient outcome by using the Mann-Whitney-Wilcoxon rank-sum test for continuous variables and the χ2 test with Yates correction for categorical variables. For multivariable logistic regression, outcome variables were hospital deaths and intensive care unit (ICU) admissions, and covariates were resistance, age group, age <10 years, malnutrition, sex, and organism type (Enterobacteriaceae vs. A. baumannii). We conducted multivariable linear regression by using admission duration and cost for survivors as outcome variables and using the same covariates. The linear model variables were log transformed, and results are presented with log- and back-transformed coefficients, which is interpreted as a multiplicative rather than an additive model. Analyses were undertaken by using the R statistical package (15).

Results
During the 10-year study period, 39,050 sterile site samples were collected for culture: 36,358 (93.1%) blood and 2,692 (6.9%) CSF (online Technical Appendix Figure 1). The sampling rate, indicated by the blood culture:hospital admission ratio, rose throughout the study period as utility of the clinical microbiology service increased. Approximately 1 blood culture was sent for every 3 admissions in 2007 (1,293 blood cultures:3,829 admissions), rising to 1 blood culture per admission in 2013 (5,294 blood cultures:5,208 admissions) and subsequently remaining stable (online Technical Appendix Figure 2). From 2012 through 2016, the proportion of blood cultures from neonates rose from 9.1% to 21.2%, and the proportion from children ≥5 years of age dropped from 35.4% to 22.4% (online Technical Appendix Table 2).

Of the 39,050 specimens collected, 3,666 (9.4%) were culture positive, yielding 4,028 isolates. Skin organism contamination was identified in 1,937 (5.3%) blood cultures. Clearly pathogenic bacteria comprised 37.5% (1,512) of isolates grown, 9.1% (366) were of uncertain significance,
and 53.4% (2,150) were designated skin contaminants. A total of 1,341 target organisms met inclusion criteria; 1,088 (81.1%) were GLASS pathogens and 253 (18.9%) were non-GLASS pathogens. GLASS pathogens were Salmonella spp. (408, 30.4%); S. aureus (186, 13.9%); S. pneumoniae (166, 12.4%); K. pneumoniae (146, 10.9%); E. coli (107, 8.0%); and A. baumannii (75, 5.6%).

**Overall AMR Rates**

Overall AMR rates were high, especially among gram-negative GLASS organisms (Table 1). Ampicillin–gentamicin resistance (resistance to both agents) was detected in 62.1% (90/145) of K. pneumoniae isolates and 47.2% (50/106) of E. coli isolates. Third-generation cephalosporin resistance was detected in 78.8% (115/146) of K. pneumoniae isolates, 49.5% (53/107) of E. coli isolates, and 93.3% (70/75) of A. baumannii isolates; multidrug resistance in these 3 organisms was 81.8% (108/132), 82.1% (69/84), and 93.3% (70/75), respectively. Carbapenem resistance was uncommon: <1% of K. pneumoniae (1/142) and E. coli isolates (0/98) and 13.5% (10/74) of A. baumannii isolates were resistant.

Resistance differed greatly among the 3 groups of Salmonella spp. The proportion of resistant isolates was highest for Salmonella Typhi: 95.7% (308/322) were fluoroquinolone resistant, 86.0% (270/314) multidrug resistant, and 85.0% (266/313) fluoroquinolone and multidrug resistant. The least resistant group was Salmonella Paratyphi A: 22.7% (10/44) of isolates were fluoroquinolone resistant and none were multidrug resistant (0/43).

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td><strong>Ampicillin–gentamicin</strong></td>
<td></td>
<td>48/53 (89.3)%</td>
<td>26/30 (86.6)%</td>
<td>18/21 (85.7)%</td>
<td>13/15 (86.7)%</td>
<td>11/13 (84.6)%</td>
</tr>
<tr>
<td><strong>Ampicillin</strong></td>
<td></td>
<td>17/20 (85.0)%</td>
<td>15/18 (83.3)%</td>
<td>8/9 (88.9)%</td>
<td>7/8 (87.5)%</td>
<td>5/6 (83.3)%</td>
</tr>
<tr>
<td><strong>Carbapenem</strong></td>
<td></td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td><strong>Multidrug</strong></td>
<td></td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td><strong>Acinetobacter baumannii</strong></td>
<td></td>
<td>26/27 (96.3)%</td>
<td>22/24 (91.7)%</td>
<td>15/15 (100)%</td>
<td>10/10 (100)%</td>
<td>7/7 (100)%</td>
</tr>
<tr>
<td><strong>Salmonella Paratyphi A</strong></td>
<td></td>
<td>35/36 (97.2)%</td>
<td>23/24 (95.8)%</td>
<td>17/17 (100)%</td>
<td>14/14 (100)%</td>
<td>9/9 (100)%</td>
</tr>
<tr>
<td><strong>Salmonella Typhi</strong></td>
<td></td>
<td>31/32 (96.8)%</td>
<td>26/27 (96.3)%</td>
<td>19/19 (100)%</td>
<td>14/14 (100)%</td>
<td>11/11 (100)%</td>
</tr>
</tbody>
</table>

*Resistance proportions are reported as no. resistant isolates/no. isolates tested. Blank cells indicate that no organisms were tested during that period and, thus, the proportion of resistant organisms is unknown. 3GC, third-generation cephalosporin; AMP–GEN, resistance to both ampicillin and gentamicin; CRO, ceftazidime; FQ, fluoroquinolone; MDR, multidrug resistant; MAC/LIN, resistance to macrolides and/or lincosamides; MET, methicillin; VAN, vancomycin. 

†K. pneumoniae is intrinsically resistant to AMP, and thus AMP–GEN resistance in K. pneumoniae isolates is equivalent to GEN resistance.
Resistance in non-typhoidal *Salmonella* spp. fell between that of *Salmonella* Typhi and Paratyphi A: 63.4% (26/41) of isolates were fluoroquinolone resistant, 23.1% (9/39) multidrug resistant, and 12.8% (5/39) fluoroquinolone and multidrug resistant. Only 1.6% (4/254) of *Salmonella* spp. isolates were ceftriaxone resistant.

In gram-positive GLASS organisms, approximately one third of *S. pneumoniae* isolates were macrolide/lincosamide resistant (29.7%, 49/165), half were penicillin resistant (50.7%, 73/144), and two thirds were multidrug resistant (37.1%, 13/35), followed by *Enterobacter cloacae*, which had a similar resistance profile to *K. pneumoniae* and *E. coli*. The remaining non-GLASS pathogens (group A *Streptococcus*, *Pseudomonas aeruginosa*, and *Neisseria meningitidis*) exhibited low-level resistance to the key antimicrobials reported.

**AMR Time Trends**

The most frequently isolated organisms were *K. pneumoniae*, *E. coli*, *Salmonella* Typhi, *S. aureus*, and *S. pneumoniae*: AMR time trends were heterogeneous (Tables 1, 3–5; Figure 1). *S. pneumoniae* penicillin resistance fluctuated over time; 55.6% (5/9) of isolates were resistant in 2007–2008, dropping to 41.0% (16/39) in 2011–2012 before rising to 68.8% (22/32) in 2015–2016. During 2011–2016, when we tested *S. pneumoniae* for multidrug resistance, the proportion of multidrug-resistant *S. pneumoniae* isolates increased from 50.0% to 84.4%.

*Salmonella* Typhi fluoroquinolone resistance also increased over the study period, from 88.6% to 100%. Multivariable logistic regression analysis in which time was a continuous variable showed an increased probability of *Salmonella* Typhi fluoroquinolone resistance over time (adjusted odds ratio [aOR] 2.14, 95% CI 1.29–3.74; *p* = 0.005), although not statistically significant in the model when time was a factor (online Technical Appendix Table 3 and Figure 3). Conversely, during 2015–2016, the proportion of resistant *K. pneumoniae* isolates fell dramatically for most antimicrobials tested, a phenomenon not seen for *E. coli* (online Technical Appendix Table 4). For *E. coli*, ampicillin–gentamicin and third-generation cephalosporin resistance remained stable at ≤50% with no evidence of significant change over time, as did rates of methicillin-resistant *S. aureus*, which remained low throughout the study period. The proportion of *K. pneumoniae* isolates from neonates peaked in 2011–2014 at 46%–50% before dropping in 2015–2016 to 35% (online Technical Appendix Table 5), paralleling the change in the proportion of resistant isolates seen. To determine any subtle shifts in susceptibility, we examined changes in zone diameter distribution over time for *E. coli* and *K. pneumoniae* and found no clear trends (online Technical Appendix Table 6, Figures 4, 5).

**Table 2.** Resistance proportions by year of isolation for the 253 non-Global Antimicrobial Resistance Surveillance System pathogens isolated from children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016*

<table>
<thead>
<tr>
<th>Pathogen, resistance type</th>
<th>No. isolates</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia pseudomallei</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAZ</td>
<td>0/66</td>
<td>0/60</td>
</tr>
<tr>
<td>TMP/SXT</td>
<td>0/61</td>
<td>0/2</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>30/56 (53.6)</td>
<td>5/14 (35.7)</td>
</tr>
<tr>
<td>CRO</td>
<td>3/57 (5.3)</td>
<td>1/15 (6.7)</td>
</tr>
<tr>
<td>Multidrug</td>
<td>13/35 (37.1)</td>
<td>0/1</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP–GEN</td>
<td>19/42 (45.2)</td>
<td>1/2 (50.0)</td>
</tr>
<tr>
<td>3GC</td>
<td>34/42 (81.0)</td>
<td>1/2 (50.0)</td>
</tr>
<tr>
<td>Carbapenem</td>
<td>3/41 (7.3)</td>
<td>0/1</td>
</tr>
<tr>
<td>Multidrug</td>
<td>18/37 (48.6)</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td><em>Group A Streptococcus</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAC/LIN</td>
<td>6/37 (16.2)</td>
<td>0/2</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAZ</td>
<td>4/34 (11.8)</td>
<td>0/4</td>
</tr>
<tr>
<td>Carbapenem</td>
<td>2/30 (6.7)</td>
<td>0/1</td>
</tr>
<tr>
<td>Multidrug</td>
<td>0/29</td>
<td>0/0</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRO</td>
<td>1/13 (7.7)</td>
<td>0/6</td>
</tr>
</tbody>
</table>

*Resistance proportions have been reported as number of resistant isolates out of number of isolates tested. Blank cell indicates that no organisms were tested during that period and, thus, the proportion of resistant organisms is unknown. 3GC, third-generation cephalosporin; AMP–GEN, resistance to both ampicillin and gentamicin; CAZ, ceftazidime; CRO, ceftriaxone; MAC/LIN, resistance to macrolides and/or lincosamides; TMP/SXT, trimethoprim/sulfamethoxazole.

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**AMR by Patient Age Group**
Isolates from younger children were more often resistant to clinically important antimicrobials (Tables 3–5; Figure 2; online Technical Appendix Tables 7–9). Multivariable logistic regression controlling for year of isolation and site of acquisition indicated that *K. pneumoniae* isolates from neonates were >7 times more likely to be resistant to the first-line treatment agents ampicillin–gentamicin.

### Table 3. Logistic regression analysis of resistance trends for the gram-negative Global Antimicrobial Resistance Surveillance System pathogens *Klebsiella pneumoniae* and *Escherichia coli* isolated from children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016*

<table>
<thead>
<tr>
<th>Pathogen, resistance type, predictor variable</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>†Neonate, 0–28 d of age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007–2008</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2011–2012</td>
<td>3.61 (0.94–12.43)</td>
<td>1.12 (0.29–4.40)</td>
</tr>
<tr>
<td>2013–2014</td>
<td>0.61 (0.16–2.36)</td>
<td>0.24 (0.05–1.16)</td>
</tr>
<tr>
<td>2015–2016</td>
<td>1.02 (0.30–3.57)</td>
<td>0.52 (0.12–2.32)</td>
</tr>
<tr>
<td>Patient age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonneonate</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Neonate†</td>
<td>2.61 (1.17–5.80)</td>
<td>1.31 (0.65–2.62)</td>
</tr>
<tr>
<td>Hospital-acquired</td>
<td>1.83 (0.72–4.62)</td>
<td>0.78 (0.31–2.02)</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007–2008</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2011–2012</td>
<td>1.44 (0.56–3.67)</td>
<td>1.01 (0.40–2.57)</td>
</tr>
<tr>
<td>2013–2014</td>
<td>1.03 (0.36–3.10)</td>
<td>0.42 (0.13–1.42)</td>
</tr>
<tr>
<td>2015–2016</td>
<td>1.43 (0.95–2.14)</td>
<td>1.04 (0.60–1.82)</td>
</tr>
<tr>
<td>Patient age</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonneonate</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Neonate†</td>
<td>1.71 (0.81–3.63)</td>
<td>1.11 (0.50–2.49)</td>
</tr>
<tr>
<td>Hospital-acquired</td>
<td>1.36 (0.56–3.33)</td>
<td>0.85 (0.33–2.17)</td>
</tr>
</tbody>
</table>

---

*3GC, third-generation cephalosporin; AMP–GEN, resistance to both ampicillin and gentamicin; OR, odds ratio; ref, referent.
†Neonate, 0–28 d of age.
‡Isolates were defined as hospital-acquired if taken >48 hours after admission.
Table 4. Logistic regression analysis of resistance trends for the gram-negative Global Antimicrobial Resistance Surveillance System pathogen Salmonella enterica serovar Typhi isolated from children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016*

<table>
<thead>
<tr>
<th>Resistance type, predictor variable</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95% CI)</td>
<td>p value</td>
</tr>
<tr>
<td></td>
<td></td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007–2008</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2009–2010</td>
<td>2.05 (0.47–10.51)</td>
<td>0.345</td>
</tr>
<tr>
<td>2011–2012</td>
<td>2.97 (0.82–10.37)</td>
<td>0.085</td>
</tr>
<tr>
<td>2013–2014</td>
<td>4.03 × 10^7 (6.26 × 10^6&lt;x)</td>
<td>0.992</td>
</tr>
<tr>
<td>2015–2016</td>
<td>4.03 × 10^7 (1.11 × 10^33–&lt;x)</td>
<td>0.992</td>
</tr>
<tr>
<td>Patient age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>4.48 (0.87–82.12)</td>
<td>0.151</td>
</tr>
<tr>
<td>&lt;5</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Multidrug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2007–2008</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2009–2010</td>
<td>1.57 (0.56–4.58)</td>
<td>0.395</td>
</tr>
<tr>
<td>2011–2012</td>
<td>4.32 (1.64–11.44)</td>
<td>0.003</td>
</tr>
<tr>
<td>2013–2014</td>
<td>2.26 (0.72–7.92)</td>
<td>0.175</td>
</tr>
<tr>
<td>2015–2016</td>
<td>0.91 (0.33–2.46)</td>
<td>0.850</td>
</tr>
<tr>
<td>Patient age, y</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;5</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>&lt;5</td>
<td>2.94 (1.22–8.79)</td>
<td>0.029</td>
</tr>
</tbody>
</table>

*OR, odds ratio; ref, referent.

(aOR 7.30, 95% CI 2.75–22.47) and third-generation cephalosporins (aOR 7.50, 95% CI 2.16–35.00) than were isolates from nonneonates. Similarly, S. pneumoniae isolates were more likely to be penicillin resistant (aOR 3.87, 95% CI 1.77–8.83) and Salmonella Typhi isolates more likely to be multidrug resistant (aOR 3.16, 95% CI 1.28–9.57) among children <5 years of age than among those ≥5 years of age.

AMR by Site of Infection Acquisition

Approximately four fifths of included isolates were from community-acquired infections (1,089, 81.2%) and one fifth from hospital-acquired infections (252, 18.8%). In almost all instances, the proportion of hospital-acquired isolates resistant to a given antimicrobial was higher than that of community-acquired isolates (online Technical Appendix Tables 10–12). K. pneumoniae, the main cause of hospital-acquired infections, was >3 times more likely to be resistant to ampicillin–gentamicin (aOR 3.62, 95% CI 1.42–9.58) and third-generation cephalosporins (aOR 3.51, 95% CI 1.27–10.12) in hospital-acquired isolates (Tables 3, 4). Increased likelihood of resistance among hospital-acquired isolates was also found for E. coli ampicillin–gentamicin and third-generation cephalosporin resistance and S. aureus methicillin resistance.

Outcomes

We analyzed patient outcomes for 129 admission episodes for community-acquired monomicrobial gram-negative bacteremia (online Technical Appendix Figure 6). Of these, 63 (48.8%) isolates were resistant to third-generation cephalosporins and 34 admissions (26.4%) resulted in patient death. Isolates consisted of E. coli (48, 37.2%), K. pneumoniae (31, 24.0%), A. baumannii (29, 22.5%), and other pathogenic Enterobacteriaceae (21, 16.3%). Neonates accounted for 26.4% (34) of the cases; median age was 8.6 months (interquartile range [IQR] 0.8–29.2 months).

Children from whom third-generation cephalosporin-resistant bacteria were isolated were less likely than other patients to have received appropriate antimicrobial therapy (57% vs. 94%; p<0.001). If appropriate therapy was received, it was initiated later for children infected with third-generation–resistant than third-generation–sensitive organisms (2 days vs. 0 days after admission for those who survived [p<0.001]; 0.5 days vs. 0 days for those who died [p = 0.004]). Patients who died were younger (median age 1.4 vs. 10.2 months; p = 0.002), were more likely to have been admitted to an ICU (88% vs. 27%; p<0.001), stayed for a shorter time in hospital (3 vs. 8 days; p<0.001), and were more likely to have been infected with Enterobacteriaceae than A. baumannii (97% vs. 71%; p = 0.003) (online Technical Appendix Table 13). A. baumannii infections were associated with high levels of third-generation cephalosporin resistance (90%) but a low mortality rate (3%) despite only 48% of patients having received appropriate antimicrobials. Conversely, Enterobacteriaceae infections were associated with a high mortality rate (33%) despite 84% of patients having received appropriate antimicrobials (online Technical Appendix Table 14).

Multivariable logistic regression (Table 6) showed that third-generation cephalosporin resistance was associated with death (aOR 2.65, 95% CI 1.05–6.96; p = 0.042) and ICU admission (aOR 3.17, 95% CI 1.31–8.10; p =
Table 5. Logistic regression analysis of resistance trends for the gram-positive Global Antimicrobial Resistance Surveillance System pathogens *Staphylococcus aureus* and *Streptococcus pneumoniae* isolated from children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016*

<table>
<thead>
<tr>
<th>Pathogen, resistance type, predictor variable</th>
<th>Univariable analysis</th>
<th>Multivariable analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2007–2008</td>
<td>0.90 (0.18–4.93)</td>
<td>1.26 (0.23–7.59)</td>
</tr>
<tr>
<td>2009–2010</td>
<td>0.899</td>
<td>1.04 (0.19–5.56)</td>
</tr>
<tr>
<td>2011–2012</td>
<td>0.418</td>
<td>0.24 (0.04–1.45)</td>
</tr>
<tr>
<td>2013–2014</td>
<td>0.538</td>
<td>0.66 (0.10–4.19)</td>
</tr>
<tr>
<td>2015–2016</td>
<td>0.603</td>
<td>1.84 (0.39–10.47)</td>
</tr>
<tr>
<td>Patient age†</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Nonneonate</td>
<td>0.18 (0.01–0.88)</td>
<td>0.14 (0.01–0.75)</td>
</tr>
<tr>
<td>Neoneonate</td>
<td>0.094</td>
<td></td>
</tr>
<tr>
<td>Infection type‡</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Community-acquired</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Hospital-acquired</td>
<td>6.21 (2.16–17.43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Year of isolation</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>2007–2008</td>
<td>0.60 (0.12–2.90)</td>
<td>0.70 (0.13–3.66)</td>
</tr>
<tr>
<td>2009–2010</td>
<td>0.52 (0.11–2.28)</td>
<td>0.42 (0.08–1.95)</td>
</tr>
<tr>
<td>2011–2012</td>
<td>0.385</td>
<td>0.77 (0.16–3.57)</td>
</tr>
<tr>
<td>2013–2014</td>
<td>0.663</td>
<td>1.89 (0.36–9.59)</td>
</tr>
<tr>
<td>2015–2016</td>
<td>0.424</td>
<td></td>
</tr>
<tr>
<td>Patient age, y</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>≥5</td>
<td>3.40 (1.63–7.39)</td>
<td>3.87 (1.77–8.83)</td>
</tr>
<tr>
<td>&lt;5</td>
<td>0.001</td>
<td></td>
</tr>
</tbody>
</table>

1OR, odds ratio; ref, referent.
2Ages are grouped into neonate (0–28 d) vs. nonneonate (≥29 d) or <5 y vs. ≥5 y, as appropriate for the organism.
3Isolates were defined as hospital-acquired if taken >48 hours after patient admission.
4Analysis included community-acquired *Streptococcus pneumoniae* isolates only (n = 160).

0.013). Multivariable linear regression (online Technical Appendix Table 15) controlling for the same variables also showed an association between length of hospital stay among survivors and third-generation cephalosporin resistance (1.69-fold increase, 95% CI 1.21–2.37). Third-generation cephalosporin resistance was associated with a 2.26-fold increase in hospital costs among survivors (95% CI 1.51–3.36) (online Technical Appendix Tables 16, 17). According to this model, the median cost per admission would have been US $432.00 (IQR $333.30–$613.90) if all infections were third-generation cephalosporin sensitive and US $974.10 (IQR $751.60–$1,384.30) if all infections were third-generation cephalosporin resistant.

**Discussion**

In this hospitalized population of children in Cambodia, AMR levels were high, particularly among the gram-negative GLASS pathogens *K. pneumoniae*, *E. coli*, and *A. baumannii*. These organisms exhibited concerning resistance to WHO-recommended first-line sepsis treatment, emphasizing the urgent need for revised treatment guidelines (4). Few studies inform prevalence estimates of antimicrobial resistance in low- and middle-income countries in Asia, but compared with what is known, the high levels of gram-negative resistance reported here are not uncommon (6,16). For the gram-positive GLASS pathogens, *S. pneumoniae* resistance was broadly similar to that of the wider region (67.7% vs. 59.3% multidrug resistance, respectively) (17). Rates of methicillin-resistant *S. aureus* were comparatively lower; only 40.0% of hospital-acquired isolates were methicillin resistant compared with a regional average of 67.4% (18).

A major strength of this study is the observation of resistance trends over an extended period, something rarely possible in low- and middle-income countries because of lack of longstanding microbiology services. We found heterogeneous trends in resistance over time; resistance increased in some organisms (*Salmonella Typhi*) and decreased in others (*K. pneumoniae*). The most surprising temporal trend observed was a drop in the proportion of resistant *K. pneumoniae* isolates for most antimicrobials tested, in contrast to largely stable resistance levels in *E. coli*. For *K. pneumoniae* resistance by site of acquisition, in community-acquired isolates, resistance sharply declined in 2015–2016, perhaps suggesting loss of a plasmid coding for multiple resistance determinants. Confirming this trend will require a larger dataset from multiple sites in Cambodia and further analysis of the underlying resistance mechanisms at work using a method such as whole-genome sequencing. The genetic determinants of resistance in colonizing *K. pneumoniae* and *E. coli* isolates from
this population have been reported elsewhere (CE Moore, CM Parry, P Turner, NPJ Day, N Stoesser, unpub data; N Stoesser, C Turner, P Turner, BS Cooper, unpub data), whereas whole-genome sequencing of invasive isolates is ongoing. Loss of antimicrobial selective pressure leading to declining resistance may result from changes in national/regional antimicrobial supply or lack of active drug in antimicrobials used (19).

The number of hospital-acquired \textit{K. pneumoniae} isolates peaked during 2011–2012. This peak may be the result of a genuine rise in the rate of hospital-acquired infections or the increased rate of blood culture sampling compared with previous years. From 2011–2012 onward, the proportion of resistant hospital-acquired \textit{K. pneumoniae} isolates declined. This drop may be linked to maturation of a hospitalwide infection-control program implemented in 2010 (20) and enforced by prospective hospital-acquired infection surveillance from 2015 onward (21) or to the clinical microbiology service operating since 2012 with a strong focus on antimicrobial drug stewardship. Indeed, a recent study of prescribing practices at this hospital found 84%–89% of antimicrobial drug prescriptions were appropriate (22). The apparent success of these interventions suggests that they could be useful for combating AMR in similar settings. The perceived temporal drop in \textit{K. pneumoniae} resistance could also be attributable to changing proportions of isolates from neonates over time; 46%–50% of isolates were from neonates in 2011–2014, dropping to 35% in 2015–2016.

Figure 1. Antimicrobial resistance time trends, shown as proportion of resistant isolates from community-acquired and hospital-acquired infections, by year of isolation, in children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016. A) \textit{Klebsiella pneumoniae} ampicillin–gentamicin resistance; B) \textit{K. pneumoniae} third-generation cephalosporin resistance; C) \textit{K. pneumoniae} multidrug resistance; D) \textit{Escherichia coli} ampicillin–gentamicin resistance; E) \textit{E. coli} third-generation cephalosporin resistance; F) \textit{E. coli} multidrug resistance; G) \textit{Salmonella enterica} serotype Typhi fluoroquinolone resistance; H) \textit{Staphylococcus aureus} methicillin resistance; I) \textit{Streptococcus pneumoniae} penicillin resistance. Isolates were defined as hospital-acquired if taken >48 hours after patient admission. Error bars indicate 95% CIs.
This study is unusual in that it directly compares different age groups of children, revealing AMR trends associated with age. Of note, the dominant pathogen in neonates, *K. pneumoniae*, was also more often resistant in neonates. For hospital-acquired isolates, this resistance may result from horizontal acquisition of resistant gram-negative organisms from hospital surfaces, as suggested by a recent multicenter study of sepsis in neonates (2). Indeed, colonization of neonates by resistant gram-negative organisms has been shown to be common at Angkor Hospital for Children and associated with subsequent invasive infection (23). For community-acquired isolates, vertical maternal transfer of resistant organisms may have a substantial role and is currently under investigation at this center.

Similarly, *Salmonella Typhi* from children <5 years of age was more often multidrug resistant than that from those ≥5 years of age. Isolates from younger children have greater genetic diversity (24), although how this diversity relates to increased AMR requires further investigation. In Cambodia, the most common indication for antimicrobial drug use is infections in children <5 years of age; thus, children in this age group may be exposed to more antimicrobial drugs, leading to greater resistance in organisms causing infection. That *S. pneumoniae* isolates were more often penicillin resistant in children <5 years of age is consistent with findings of previous work showing greater colonization of this age group by multidrug-resistant pneumococci (25). Vaccination may have a collateral benefit of reducing AMR (26), which suggests that it could be useful for combating *Salmonella Typhi* and *S. pneumoniae* resistance in low- and middle-income countries. Because 85% of *Salmonella Typhi* isolates are simultaneously fluoroquinolone resistant and multidrug resistant, few agents remain for treating typhoid in this population, placing even greater value on preventive measures such as vaccination. In January 2015, a 13-valent pneumococcal conjugate vaccine was introduced in Cambodia (27) with no catch-up campaign, meaning that only *S. pneumoniae* isolates from children born in or after December 2014 could have been affected, equating to 5 isolates in this dataset. Pneumococcal vaccination is thus unlikely to have had an appreciable effect on the AMR trends reported here.

The WHO Global Report on Surveillance identified a major gap in research comparing resource use in resistant versus nonresistant pathogens (28), an area that we addressed by demonstrating that resistance is associated with worse healthcare outcomes, including increased deaths and ICU admissions, delayed effective treatment, and more than doubled admission costs. Use of patient records allows these estimates to more closely reflect reality than modeled or ecologic analyses, although it is unclear whether this increased risk for adverse outcomes represents greater virulence, delayed treatment, or confounding. The observed outcome differences between *Enterobacteriaceae* and *A. baumannii* infections suggest either a true difference in virulence or that a proportion of *A. baumannii* isolates were contaminants, an uncertainty that highlights the difficulty of establishing the clinical significance of skin-colonizing organisms.

This study has several limitations. The data derive from a single nongovernmental hospital for children with limited numbers of isolates for some bacterial species; thus, trends and outcomes may not be representative of the wider region. The study was retrospective, and classification of community-acquired and hospital-acquired infections was limited by hospital database and clinical case note accuracy, meaning that some community-acquired infections may have actually been hospital-acquired infections. Widespread prehospitalization use of antimicrobials may have selected for resistant organisms (29). There were no restrictions to blood culture submission over time, but from early 2016 onward, clinicians were asked to focus on children requiring admission, which may have affected certain

![Figure 2](image-url)
organisms (e.g., Salmonella Typhi). Microbiology practice variations over time meant that antimicrobial susceptibility testing was not consistent; however, we believe that the value of examining the evidence over a long period outweighed the effect that these variations may have had on results. For example, in 2009, the Clinical and Laboratory Standards Institute sensitivity zone size cutoffs for carbapenems and cephalosporins in Enterobacteriaceae increased, which could have resulted in a small number of isolates previously classed as sensitive being reclassified as resistant. The reported pre-2009 resistance levels are thus conservative and would not negate the downward resistance trends observed. In the outcome analysis, we did not consider prehospitalization factors, clinical diagnosis, and non-HIV/malnutrition co-occurring conditions because quantifying those could have introduced substantial reporting bias. Furthermore, our cost estimates may be higher than actual costs because we did not account for partial/shared doses and price fluctuations, suggesting that these cost estimates are most useful as a relative indication of cost burden.

In conclusion, the high rate of AMR in this setting of hospitalized children in Cambodia was associated with increased deaths and healthcare costs and threatens the effectiveness of first-line sepsis treatment. AMR represents a major threat to children’s health globally (5,6), yet there is a dearth of data for children in low-resource settings (3–6). By reporting a decade of continuous AMR surveillance data, this study fills a gap in the understanding of antimicrobial drug resistance in children in Cambodia. In the context of the current global drive to combat AMR and the goal of the Fleming Fund to improve surveillance in low- and middle-income countries, our study demonstrates the feasibility and utility of undertaking accurate long-term antimicrobial drug resistance surveillance in these countries. The methods used here are reproducible in similar low-resource settings.

Acknowledgments
We thank the clinical, laboratory, and support staff at Angkor Hospital for Children.

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About the Author
Dr. Fox-Lewis is a research clinician at the Cambodia-Oxford Medical Research Unit, Angkor Hospital for Children, Siem Reap, Cambodia. His primary research interest is antimicrobial resistance.

References

**Table 6. Multivariable logistic regression analysis of 129 hospital admission episodes for community-acquired monomicrobial gram-negative bacteremia in children at Angkor Hospital for Children, Siem Reap, Cambodia, 2007–2016**

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Death</th>
<th>p value</th>
<th>OR (95% CI)</th>
<th>p value</th>
<th>ICU admission</th>
</tr>
</thead>
<tbody>
<tr>
<td>Third-generation cephalosporin resistance</td>
<td>2.65 (1.05–6.96)</td>
<td>0.042</td>
<td>3.17 (1.31–8.10)</td>
<td>0.013</td>
<td>4.56 (1.83–12.16)</td>
</tr>
<tr>
<td>Neonate†</td>
<td>3.03 (1.14–8.31)</td>
<td>0.028</td>
<td>4.65 (1.83–12.16)</td>
<td>0.002</td>
<td>4.56 (1.83–12.16)</td>
</tr>
<tr>
<td>Male</td>
<td>0.81 (0.32–2.07)</td>
<td>0.659</td>
<td>0.81 (0.35–1.85)</td>
<td>0.616</td>
<td>0.81 (0.35–1.85)</td>
</tr>
<tr>
<td>Enterobacteriaceae‡</td>
<td>26.25 (4.43–511.1)</td>
<td>0.003</td>
<td>3.07 (1.05–9.67)</td>
<td>0.046</td>
<td>3.07 (1.05–9.67)</td>
</tr>
<tr>
<td>Malnourished§</td>
<td>2.11 (0.85–5.35)</td>
<td>0.111</td>
<td>2.19 (0.98–5.01)</td>
<td>0.059</td>
<td>2.19 (0.98–5.01)</td>
</tr>
<tr>
<td>Age &lt;10 y</td>
<td>2.76 (0.40–56.29)</td>
<td>0.377</td>
<td>2.80 (0.60–20.70)</td>
<td>0.235</td>
<td>2.80 (0.60–20.70)</td>
</tr>
</tbody>
</table>

*Analysis used outcome (death or recovery) and ICU admission as the dependent variables. ICU, intensive care unit; OR, odds ratio.†0–28 d of age.‡Acinetobacter baumannii n = 29; Enterobacteriaceae n = 100 (consisting of Escherichia coli, n = 48; Klebsiella pneumoniae, n = 31; other pathogenic Enterobacteriaceae [consisting of Citrobacter, Enterobacter, Escherichia, Klebsiella, Morganella, Pantoea, Proteus, and Serratia spp. n = 21]).§Children <10 y of age only.


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Galicia in northwestern Spain has been considered a hotspot for *Vibrio parahaemolyticus* infections. Infections abruptly emerged in 1998 and, over the next 15 years, were associated with large outbreaks caused by strains belonging to a single clone. We report a recent transition in the epidemiologic pattern in which cases throughout the region have been linked to different and unrelated strains. Global genome-wide phylogenetic analysis revealed that most of the pathogenic strains isolated from infections were associated with globally diverse isolates, indicating frequent episodic introductions from disparate and remote sources. Moreover, we identified that the 2 major switches in the epidemiologic dynamics of *V. parahaemolyticus* in the regions, the emergence of cases and an epidemiologic shift in 2015–2016, were associated with the rise of sea surface temperature in coastal areas of Galicia. This association may represent a fundamental contributing factor in the emergence of illness linked to these introduced pathogenic strains.

Globally, *Vibrio parahaemolyticus* is the leading bacterial cause of illness associated with seafood consumption. Infections have undergone a global expansion over the last 2 decades; cases have suddenly emerged in areas considered environmentally adverse for these pathogens (1–3). The causes of this dynamic expansion and emergence in non–disease-endemic areas have remained elusive. *V. parahaemolyticus* infections are generally rare and sporadic across all of Europe with a single exception: Galicia in northwestern Spain. This region has been considered a hotspot for *Vibrio* infections and an anomaly within the epidemiologic context of *V. parahaemolyticus* in Europe; recurring cases of foodborne vibriosis (4–7) and outbreaks (7,8) have been reported regularly since the late 1990s. Infections associated with *V. parahaemolyticus* in Galicia were characterized by sudden outbreaks of illness typically associated with a single genetic variant of the pathogen (6,8). The first sign of change in this epidemiologic pattern was observed in 2012 when 3 different and genetically unrelated strains of *V. parahaemolyticus* were identified during a large outbreak in Galicia (5,9). Since then, a clear transition in the epidemiology of this pathogen has been observed; sporadic cases scattered throughout the region have been caused by different and unrelated strains and typically associated with the consumption of locally produced shellfish.

We applied whole-genome sequencing for a comprehensive and high-resolution insight into pathogenic populations identified in clinical sources associated with the major episodes of illness in Galicia over the past 20 years. We performed phylogenetic analysis to identify the population structure and potential sources of the clinical strains identified in Galicia. We also conducted a parallel exploration of the variability of environmental conditions in the region to investigate the existence of other factors contributing to the emergence of illness linked to these in this particular area.

Materials and Methods

**Bacterial Strains and DNA Extraction**

We analyzed 18 isolates derived from clinical sources collected over the course of the different outbreaks in Galicia...
(Table). All the strains isolated from infections in Galicia were characteristically tdh positive and trh negative, the only exceptions being the strains belonging to sequence type (ST) 36 isolated during the 2012 outbreak, which were positive for both haemolysin genes. Additionally, we included 14 isolates obtained from environmental sources (shellfish and zooplankton) during 2003–2007 to analyze the potential connection between the clinical pathogenic populations and local marine environmental sources. Finally, we added 4 clinical strains reported in the United Kingdom associated with human infections since the 1970s to the study to explore possible connections between pathogenic populations within Europe, along with another 6 environmental strains from the United Kingdom isolated in 2014.

**Genome Sequencing and Sequence Processing**

We performed genomic DNA extraction of the 42 strains from overnight cultures using the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany). We sequenced the genomes of all 42 strains using MiSeq (Illumina, San Diego, CA, USA) with a minimum coverage of 40–120-fold. We prepared libraries with the Nextera XT DNA sample preparation kit (Illumina) and de novo assembled whole-genome sequence contigs for each strain by using CLC Genomics Workbench version 7.5.1 (QIAGEN, Valencia, CA, USA).

**Global Collection of V. parahaemolyticus Genomes**

We initially investigated the position of the strains from Spain and the United Kingdom on the global phylogeny

### Table. Characteristics of *Vibrio parahaemolyticus* strains sequenced and analyzed for study of epidemic dynamics, 1998–2016*

<table>
<thead>
<tr>
<th>Strain</th>
<th>CSFAN no.</th>
<th>Year</th>
<th>Source</th>
<th>ST</th>
<th>tdh</th>
<th>trh</th>
<th>Accession no.†</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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Strains identified in the United Kingdom

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14-692-A-1-VP CSFAN029642 2014 Shellfish ST1159 – + SRR5639915 This study
V12-024 CSFAN029651 2014 Clinical ST3 – + SRR5639912 This study
V05-002 CSFAN029650 1972 Clinical ST331 – + SRR5639911 This study
V06-002 CSFAN029649 1980 Clinical ST17 – + SRR5639918 This study
F3305-VP CSFAN029648 2005 Clinical ST262 – + SRR5639917 This study

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*CSFAN, Center for Food Safety and Applied Nutrition; NA, not applicable; SRA, Sequence Read Archive; ST, sequence type; †, positive; ‡, negative.
†National Center for Biotechnology Information Assembly or Sequence Read Archive database.
of *V. parahaemolyticus* using all the available *V. parahaemolyticus* genomes worldwide, including 696 genomes obtained from the National Center for Biotechnology Information Assembly and Sequence Read Archive (SRA) databases (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/5/17-1700-Techapp1.pdf) plus the 42 genomes sequenced in our study. We transformed the SRA data to fastq using SRA Toolkit (fastq-dump –split-files –gzip –skip-technical) (https://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi?view=toolkit_doc). We performed genome assembly with A5-pipeline (10). We performed in silico inference of MLST profiles and STs using MLST software (https://github.com/tseemann/mlst), which infers STs using the public MLST scheme for *V. parahaemolyticus* based on 7 housekeeping genes (https://pubmlst.org/vparahaemolyticus/).

**Nucleotide Sequence Accession Numbers**

The draft genome sequences of all 44 *V. parahaemolyticus* strains from our study are available in GenBank. Accession numbers are provided in the Table.

**Results and Discussion**

Analysis of the 738 *V. parahaemolyticus* genomes resulted in a core genome alignment of 292,750 bp containing 12,399 SNPs. Positions of the Spanish strains in the global phylogeny (Figure 1) revealed a complex epidemiologic scenario with the existence of multiple, highly diverse genomic variants of strains associated with infections in the region. Moreover, we identified 12 different STs among clinical strains isolated over the past 2 decades (Figures 1, 2). We selected the genomes that clustered together with the genomes from Spain (115 genomes) and included them in a high-resolution phylogenetic reconstruction (Figure 2). The basis for the reconstruction was a core genome alignment of 3,049,195 bp containing 202,859 SNPs.

The original strain that was isolated over the course of the earliest documented large outbreak in Galicia in 1998–1999 (ST17), which pulsed-field gel electrophoresis (PFGE) subsequently reported as a new local clone (6), turned out to be closely related to strains previously isolated in Thailand (2006) and the United States (2006) when assessed by whole-genome phylogeny. The clinical strain from the United Kingdom isolated in Maidstone in the late 1970s (National Collection of Type Cultures no. 11344) and reported as genetically related to the Galician strains by PFGE (6), was also found to cluster with this group, with a difference of 450 SNPs.

Strains belonging to the so-called Asian pandemic clone (clonal complex [CC] 3) were first reported in Galicia in association with a large outbreak in 2004 (8). Epidemiologic analysis of the outbreak traced back the origin of the outbreak to a facility located in the international seaport of A Coruña in Galicia, suggesting that the most probable source of the pandemic strain was the discharge of ballast water carried in ships. No strain from this group was identified in Spain until summer 2016, when we identified 2 strains isolated in July from 2 independent outbreaks in the cities of Silleda and Pontevedra, investigated in 2 different hospitals, as belonging to CC3. Genomic analysis of the strains from Galicia, along with 21 additional genomes belonging to the ST3 strains isolated in other countries, resulted in a core alignment of 3,560,214 bp for the ST3 clade containing 384 SNPs. Whole-genome phylogeny revealed that the 2 strains from Galicia isolated in 2016 belonged to 2 different groups; both are different from the strain identified in Galicia in 2004. We identified strain N314 as part of the Asian group of CC3 and strain CFSAN056086 in the American group. We were able to clearly distinguish these...
2 strains from the strains isolated from the 2004 outbreak, which was identified in a separate branch closer to different Asian strains. Of note, while the 2004 outbreak was associated with imported seafood and unsafe food manipulation (8), the recent infections caused by CC3 strains were unequivocally associated with local shellfish (razor clams and cockles); compelling evidence of several successful introduction events of these strains into the marine environment of Galicia.

In many ways, the 2012 outbreak in Spain (5,13) represented a clear change in the epidemic dynamics of *V. parahaemolyticus* in the region. First, this outbreak was the largest reported across Europe linked to local seafood; second, it was the earliest known evidence of a cross-continental spreading of the ST36 clone, which is endemic to the Pacific Northwest (PNW) of the United States and one of the most virulent ST groups (13). Genomic analysis of the 92 available genomes of the ST36 isolated from

![Figure 1. Phylogenetic reconstruction of *Vibrio parahaemolyticus* based on 738 available genomes. Red dots indicate isolates from Spain collected over the past 20 years from clinical settings and environmental sources. Colors represent sequence types, and areas without color correspond to undetermined sequence types. Scale bars represent nucleotide substitutions per site.](image)
Figure 2. Phylogeny of *Vibrio parahaemolyticus* isolates from Galicia, Spain. A) Phylogenetic inference of the 42 genomes from Spain identified in this study (red text) along with all other genomes identified in the same clusters by the global phylogeny with their corresponding sequence types (STs). B) Phylogenetic tree of genomes belonging to ST3 (pandemic clone). C) Phylogenetic tree of genomes included in ST36 in the global phylogeny. Gray dots indicate bootstrap values supporting the nodes; dot sizes indicate 80% (smallest) to 100% (largest). Values <80% are not shown. Scale bars represent nucleotide substitutions per site. ND, not determined.
areas of endemicity for this group in the PNW identified a core alignment of 3,310,986 bp comprising 1,596 SNPs. Phylogenetic analysis of the ST36 lineage revealed the existence of 2 different clusters within this group (Figure 2, panel C): a first cluster composed of old strains from Canada isolated before 2005 and the United States before 2000, and a second cluster with modern representatives from the United States and Canada. This particular population structure suggests the existence of a lineage replacement in the PNW coast and western Canada, where only strains belonging to the second cluster were identified from 2005 onward. Surprisingly, we unequivocally identified the strains in the 2012 Galicia outbreak as belonging to the first cluster composed of strains extinct in their original location along the PNW coast, which suggests an early introduction of these strains into waters of Galicia and Europe (14). Furthermore, we identified a single strain from Canada as closely related to the genomes from the Galician strains with a minimum difference of 20–22 SNPs in an alignment of 3,310,986 bp, whereas variations among genomes of the ST36 strains from Galicia were 0–19 SNPs. The low level of variation among all the genomes in this clade supports a hypothesis that Galician strains originated in British Columbia, Canada, and were introduced in Galicia sometime after 2001.

A second singularity of the 2012 outbreak, and probably more noteworthy, was the fact that infections from a single outbreak were associated with several unrelated strains of *V. parahaemolyticus*. We identified 2 additional strains different from ST36 from clinical cases over the course of this outbreak, ST1031 and ST1032; both represent novel STs not reported before the 2012 outbreak. Whole-genome phylogeny of these new STs grouped these strains into 2 distinctive clusters. Strain G32, belonging to ST1032, showed a close association with several strains isolated from zooplankton in offshore waters of Galicia in 2006–2007, which could be preliminary evidence of a local origin of these strains introduced by the incursion of offshore oceanic waters. Strain G33, belonging to ST1031, was included in a single group along with strains also associated with local shellfish and isolated over the course of the outbreaks in the summers of 2015 and 2016.

Finally, we identified an additional group of strains belonging to ST327 associated with illnesses over the summer of 2015 and 2016. We included these strains in the same cluster as 1 strain from Thailand isolated in 1990 (Figure 2).

Our genome-wide phylogenetic analysis of pathogenic *V. parahaemolyticus* in northern Spain has provided novel insights into the epidemiology of *V. parahaemolyticus* in nondendemic areas. The primary result is that the study revealed the existence of a complex epidemiologic context characterized by the existence of multiple highly diverse strains, most originating far away, that caused infections associated with locally produced shellfish; this finding could be considered evidence of multiple events of introduction of foreign variants into Galicia. The source of these strains into Galicia is elusive and remains an area of ongoing interest, but we did identify through this study and previous work the 2 proposed mechanisms for dissemination of pathogenic strains: ballast water (15–17) and zooplankton migration (18,19). Ballast water has been proposed as a main source of pathogenic *Vibrio* bacteria (16) and was suggested as the mechanism of introduction of pandemic strains in the 2004 outbreak (8). In addition, we showed new evidence that unequivocally identified environmental transport through offshore zooplankton as one of the routes of introduction of new pathogenic variants via ocean currents (18–20). However, we cannot rule out the introduction of foreign mollusks into the marine water of Galicia as a possible source of new variants of pathogenic *Vibrio* bacteria from disparate and remote sources because of the magnitude of the shellfish trade in the region; the importation of shellfish from other geographic areas is a common practice to supply the high demand for products. A recent study analyzing the population structure and evolution of the ST36 clone suggests that the importation of clams from the PNW to Spain circa 2000 is the probable source of ST36 strains (14). Moreover, 2 other recent studies tracking the routes of introduction of the Manila clam from its original place of distribution in the Indo-Pacific region to Europe has also linked the origin of clam populations introduced in Spain to the PNW of the United States (area of endemicity of ST36 populations) during the importations of clams in the mid- and late 1990s (21,22). These findings closely correspond with the results shown in our study.

An underlying finding of our study is that the introduction of these highly pathogenic strains into a region is not sufficient by itself to initiate an epidemic; the introduced strains appear established in the area for a substantial period without evidence of associated illness, which suggests additional cofactors in infections and risk. Because seawater temperature has been identified as a critical factor governing the emergence of *Vibrio* diseases (1–3), we conducted an analysis of historical records of SST in the region. Results from these analyses revealed a significant trend of increased SST in the area, which followed a stepwise and incremental trend, rather than the expected linear change. We identified shifts in both the mean level of fluctuations and the variance of SST time series. We recognized 2 clear shifts of SST with a significant statistical support over the study period: June 1994, an SST increase of 0.4°C; and June 2014, an SST increase of 0.7°C (Figure 3). These 2 shifts in seawater temperature showed a close correspondence with the epidemic dynamics of *V. parahaemolyticus* in the area, showing a period with no infections before the first regime shift, a second period when the first epidemic events were identified, and finally a third period after the last regime shift in 2014, which
was concurrent with the change in the epidemiology of *V. parahaemolyticus* we report in this study. Previous data have shown that regime shift warming has led to an increase in prevalence of *Vibrio* bacteria in the environment (1,23), and epidemiologic studies on the emergence of *Vibrio* infections have identified SSTs >18°C as a critical threshold for triggering infections and substantially increasing the number of reported clinical cases (2). Analysis of the number of days with SSTs >18°C in Galicia over the past 36 years identified an increase of 1 day/year (Figure 2, panel B), which resulted in an increase of 35 days for the risk period of *V. parahaemolyticus* infections for the whole period. These results contrast with the situation in some areas of natural endemicity for pathogenic *V. parahaemolyticus*, such as the PNW for the ST36, where seawater temperatures have remained remarkably stable over the past 2 decades and regime shifts have not been detectable. The average annual SST in areas of Puget Sound in the PNW is ~5°C lower than in Galicia, showing a warming trend almost 2 orders of magnitude smaller and SST values always <18°C, according to the records of the satellite mesoscale SST time series (data not shown).

This study highlights the utility of whole-genome sequencing as a tool to elucidate key features of the transmission and potential sources of pathogenic environmental bacteria such as *Vibrio* spp. The concomitant introduction of foreign *Vibrio* variants with a significant warming trend in the region, coupled with the consumption of locally produced shellfish in the region, may be major contributing factors for the emergence of infections in Galicia. Parallel circumstances may also drive disease emergence in other areas of the world with similar environmental conditions, such as the Pacific Northwest (24,25) and the Atlantic Northeast (14,26) in the United States or the south of Chile (27). In these areas, the presence of imported *Vibrio* strains has been frequently reported associated with outbreaks, particularly during and after warming events (28). These areas represent major contributors to the escalation and global expansion of *V. parahaemolyticus* illnesses associated with the dissemination of the preeminent pathogenic clones of these organisms.

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Figure 3. Recent environmental warming trends in Galicia, Spain, 1982–2016. Trends in the mean values of SST were estimated using daily SST data from a coastal area defined by the coordinates 42°–43°N and 8.5°–9.5°W. A) Mean SST records show stepwise changes rather than a linear pattern. Two regime shifts occurred in June 1994 (0.4°C warming) and June 2014 (0.7°C), which correspond with the first emergence of *Vibrio parahaemolyticus* cases and the epidemiologic shifts observed for 2015 and 2016. B) Number of days with SST >18°C (blue dot), 5-year moving average (blue line), and regression line (yellow line); slope is of ~1 d/y (e.g., gaining 1 d/y). C) Mean SST data for Galicia for 2 periods, demonstrating the generalized warming and expansion of season with favorable conditions for sustaining *Vibrio* organisms in the environment and hence increasing risk of infection. SST, sea surface temperature.
Vibrio parahaemolyticus Illness in a Hotspot

About the Author
Dr. Martinez-Urtaza is a senior scientist at the Centre for Environment, Fisheries and Aquaculture Science in Weymouth, United Kingdom. His work covers aspects of molecular epidemiology and effects of climate on infectious diseases with research interests focused on the study of foodborne and waterborne diseases.

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Dynamics of Spirochetemia and Early PCR Detection of *Borrelia miyamotoi*

Lyudmila Karan, Marat Makenov, Nadezhda Kolyasnikova, Olga Stukolova, Marina Toporkova, Olga Olenkova

We investigated whether *Borrelia miyamotoi* disease can be detected in its early stage by using PCR for borrelial 16S rRNA, which molecule (DNA or RNA) is the best choice for this test, and whether spirochetes are present in blood during the acute phase of *B. miyamotoi* disease. A total of 473 patients with a suspected tickborne infection in Yekaterinburg, Russia, in 2009, 2010, and 2015 were enrolled in this study. Blood samples were analyzed by using quantitative PCR or ELISA, and a diagnosis of borreliosis was confirmed for 310 patients. For patients with erythema migrans migrants, 5 (3%) of 167 were positive for *B. miyamotoi* by PCR; for patients without erythema migrans, 65 (45%) of 143 were positive for *B. miyamotoi* by PCR. The median concentration for RNA was 3.8 times that for DNA. Median time for detection of *B. miyamotoi* in blood was 4 days.

*B. miyamotoi* infection usually manifests as an influenza-like disease causing high fever, headache, and myalgia but, in most instances, without erythema migrans (3). *B. miyamotoi* produces glycerophosphodiester phosphodiesterase (glpQ), which is absent in *B. burgdorferi* sensu lato and is therefore useful for serologic discrimination between relapsing fever and Lyme borreliosis. ELISAs and confirmatory Western blot assays of human serum samples have been used to detect antibodies against *B. miyamotoi* in the Netherlands (13) and the United States (14). Wagemakers et al. (15) detected antibodies against variable small protein 1 and variable large proteins (Vlp15, Vlp16, and Vlp18) in blood samples of patients with *B. miyamotoi* disease. PCR and thin and thick blood smears of peripheral blood stained with Wright stain or Giemsa were used to detect infection in patients suspected of having relapsing fever (16).

Unlike DNA, which is usually present as a single copy per cell, or mRNAs, which constitute the minor population of total cellular RNA, each bacterium contains hundreds to thousands of 16S rRNA molecules during the growth phase. Therefore, we hypothesized that an assay based on PCR amplification of cDNA molecules, representing highly and consistently transcribed *Borrelia* genes such as 16S rRNA, could improve the sensitivity of detection of *Borrelia* spp. Backstedt et al. (17) reported that leptospiral RNA–based quantitative PCRs (qPCRs) for human blood were \( \geq 100 \)-fold more sensitive than a DNA-based approach. Furthermore, detection of 16S rRNA (e.g., by nucleic acid sequence–based amplification) can distinguish viable from nonviable bacterial cells (18).

Hematogenous dissemination is a major pathogenetic event in Lyme borreliosis. Investigations of level and duration of spirochetemia caused by *B. miyamotoi* are needed for understanding the pathogenesis of the infection, as well as development of new diagnostic tools.

Our study had 3 goals. First, we sought to determine whether *B. miyamotoi* disease can be detected in its early stage by using PCR for borrelial 16S rRNA. Second, we assessed which molecule (DNA or RNA) is the best choice for this test. Third, we investigated the dynamics...
of *B. miyamotoi* spirochetemia during the acute phase of the disease.

**Methods**

**Study Design**

Patients suspected of having tickborne diseases in Russia are hospitalized if they have been bitten by a tick and show development of signs and symptoms of acute infection (fever, chills, headache, fatigue, muscle aches) within a few weeks. A diagnosis of acute Lyme borreliosis was determined by the presence of erythema migrans, detection of borrelial IgM and IgG seroconversion, and detection of pathogen DNA or RNA. In this study, the case definition for *B. miyamotoi* disease was based only on PCR results. We have previously reported detailed information about case definitions for *B. miyamotoi* disease (3).

A total of 459 patients admitted to Municipal Clinical Hospital No. 33 (Medical Association Novaya Bolnitsa) in Yekaterinburg, Russia, during May–August 2009 and June–July 2010 for suspected tickborne infection were enrolled in this study. We obtained blood samples from all patients during the first 3 days of hospitalization for analysis by qPCR and ELISA. We performed PCR for detection of 16S rRNA of *B. miyamotoi*, *B. burgdorferi* sensu lato, *Ehrlichia chaffeensis*, and *E. muris*; the 5′-untranslated region gene of tickborne encephalitis virus (TBEV); and the major outer membrane protein 2 gene of *Anaplasma phagocytophilum* as described (3).

We tested serum samples for borreliar IgM and IgG and TBEV IgM by ELISA (EUROMMUN AG, Lubeck, Germany). We estimated the number of copies of *B. miyamotoi* and *B. burgdorferi* sensu lato DNA and RNA in PCR-positive patient blood samples by using a qPCR for 16S rRNA.

For determining duration of spirochetemia, we collected repeat blood samples from 9 PCR-positive *B. miyamotoi* disease patients in 2010 and 14 *B. miyamotoi* disease patients admitted to Municipal Clinical Hospital No. 33 in June–July 2015. We collected blood samples from these patients during the first 3 days after admission and then repeatedly for 4 days after initial detection of *B. miyamotoi* in the blood of these patients.

**Preparation of Blood Samples**

We used differential centrifugation to separate spirochetes from erythrocytes. Blood samples were centrifuged at 160 × g for 10 min to pellet erythrocytes. We then transferred 500 μL of leukocytes and platelet-rich plasma into 1.5-mL tubes for centrifugation at 10,000 × g for 10 min to collect leukocytes and spirochetes. We extracted DNA and RNA from the pellet and 100 μL of the supernatant by using an AmpliSens Riboprep Kit (Central Research Institute of Epidemiology, Moscow, Russia). After cell lysis, we divided samples into 2 equal aliquots for separate isolation of *B. miyamotoi* RNA and DNA targets of 16S rRNA. We extracted RNA by using a universal internal RNA recombinant control having a known number of RNA copies per milliliter and DNA by using a universal internal DNA recombinant control having a known number of DNA copies per milliliter. RNA samples were not treated with DNase; therefore, RNA concentrations in this article are RNA/DNA concentrations.

**Molecular Detection of Infection**

We performed PCR-based detection for TBEV, *B. burgdorferi* sensu lato, *A. phagocytophilum*, *E. chaffeensis*, and *E. muris* by using a commercial multiplex PCR kit (AmpliSens TBEV, *B. burgdorferi* sensu lato, *A. phagocytophilum*, *E. chaffeensis/E. muris*-FL; Central Research Institute of Epidemiology) according to the manufacturer’s instructions. We used the *B. miyamotoi*–specific primers Brm1 5′-CGGTGAAAGCATGACACTTTTGTTGTAATC-3′ (forward) and Brm2 5′-CGGCAGTCTCTGTCTGAGTCCCCCATCT-3′ (reverse) at concentrations of 360 nmol/L. The corresponding dye-labeled probe (final concentration 100 nmol/L) used was Brm-R6G-5′-CTTGGGAGTATGTTCGCAAGAATGACTC3′-BQH1. PCR conditions were 50°C for 15 min; 95°C for 15 min; 10 cycles at 95°C for 20 s, 67°C for 50 s, and 72°C for 20 s; and 40 cycles at 95°C for 20 s, 60°C for 50 s, and 72°C for 20 s. The fluorescence signal was recorded at the 60°C step for the last 40 cycles. Each run included negative controls and 2 positive recombinant DNA controls (a *B. miyamotoi* 16S rRNA gene fragment and an internal control having 104–106 copies/mL) as standards.

We used *B. burgdorferi* sensu lato–specific primers Brb1 5′-TGCAATGCTAACAGGGATGATGCAATACA-3′ (forward) and Brb2 5′-GGTTTCCTTTCACTAATGAAACAA-3′ (reverse) at concentrations of 360 nmol/L. The corresponding dye-labeled probe (final concentration 100 nmol/L) was Brb-R6G-5′-TAGGTAGAT-BQH1CATCCACGCGTTTACCT-3′. PCR conditions were 50°C for 15 min; 95°C for 15 min; and 45 cycles at 95°C for 10 s and 60°C for 20 s. The fluorescence signal was recorded at the 60°C step for the last 45 cycles. Each run included negative controls and 2 positive recombinant DNA controls (a *B. burgdorferi* sensu lato 16S rRNA gene fragment and an internal control having 104–106 copies/mL) as standards.

The RNA template was subjected to reverse transcription (Thermo Fisher Scientific, Waltham, MA, USA). In 2015, a commercial PCR kit (AmpliSens *B. miyamotoi*-FL; Central Research Institute of Epidemiology) was used according to the manufacturer’s instructions for *B. miyamotoi* glpQ gene screening of patients with suspected tickborne diseases.
ELISA
We tested serum samples collected at the time of hospitalization and 1–2 weeks later for borrelial IgM and IgG. We obtained serologic evidence of exposure to *Borrelia* spp. by using 2 ELISAs (EI 2132–9601 M and EI 2132–9601–2 G; EUROIMMUN AG) and detected TBEV IgM by using a semiquantitative ELISA (EI 2661–9601 M; EUROIMMUN AG).

Data Analysis
We used the Wilcoxon signed-rank test for comparing copy numbers of *B. miyamotoi* RNA and DNA in blood samples and the Kaplan–Meier estimator to assess persistence of DNA or RNA of pathogens in blood. We performed time-to-event analysis by using the following assumptions: 1) starting time was the first day of illness; 2) observations for no antimicrobial drug treatment were complete data; 3) observations for late antimicrobial drug treatment (when DNA or RNA were eliminated from blood before start of treatment) were complete data; and 4) observations for antimicrobial drug treatment were incomplete data (right censored). The Kaplan–Meier estimator assumes that at any time, patients who are censored have the same survival prospects as those who continue to be followed up (19).

We used the Clopper–Pearson interval for calculating CIs for proportions. Descriptive statistics are given as mean and SD or median and interquartile range (IQR). We analyzed data by using SPSS software (IBM, Armonk, NY, USA) or R software (https://www.r-project.org/).

Results

Study Population
During 2009–2010, of 310 (67.5%, 95% CI 63.0%–71.8%) patients with borreliosis, 34 (7.4%, 95% CI 5.2%–10.2%) patients were positive for TBEV, and 115 (25.1%, 95% CI 21.2%–29.3%) were positive for other inflammatory diseases. Genetic markers of anaplasmosis and ehrlichiosis were not detected.

Table 1. Cross-validation of detection of *Borrelia* spp. by quantitative PCR and ELISA in blood samples from 310 patients with suspected borreliosis, Yekaterinburg, Russia, 2009 and 2010

<table>
<thead>
<tr>
<th></th>
<th>With erythema migrans</th>
<th>Without erythema migrans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. patients</td>
<td>No. ELISA positive</td>
</tr>
<tr>
<td>Quantitative PCR for 16S rRNA gene</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em> sensu lato</td>
<td>31</td>
<td>30</td>
</tr>
<tr>
<td><em>B. miyamotoi</em></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>PCR negative</td>
<td>131</td>
<td>81</td>
</tr>
<tr>
<td>Total</td>
<td>167</td>
<td>116</td>
</tr>
</tbody>
</table>

*ELISA results are for borrelial IgM or IgG seroconversion. ND, not determined.
disease and hospitalization was 6 days (IQR 3–9 days). We showed that PCR diagnosis of Lyme disease has low sensitivity. Consequently, *B. burgdorferi* sensu lato RNA and DNA concentration varied; the median RNA concentration was 585.0 copies/mL (IQR 305.3–1,392.5 copies/mL), and the median DNA concentration was 19.9 copies/mL (IQR 8.1–121.2 copies/mL). The concentration of *B. burgdorferi* sensu lato RNA was also significantly higher than that for DNA (p<0.001), which resulted in a greater RNA:DNA ratio (median 40.9, IQR 13.3–77.4). Differences between *B. miyamotoi* and *B. burgdorferi* sensu lato for concentrations of RNA and DNA were significant (z = -5.77, p<0.001, by Mann-Whitney U-test).

*B. miyamotoi* DNA or RNA were detected in blood samples up to day 30 of disease (Figure 2, panel A). However, despite such a wide range, 90% of all observations were in the first 8 days of the disease. We showed in a previous study (20) that 7 (9.1%) positive samples, which were obtained from patients given a diagnosis of *B. miyamotoi* disease during the second week of disease (or later), were assumed to be caused by a relapse of fever. However, this assumption could not be confirmed because of lack of availability of clinical materials. Pathogen DNA or RNA were detected in blood samples from patients with Lyme borreliosis obtained up to day 24 of disease (Figure 2, panel B).

The study showed a high number of false-negative PCR results in patients with and without EM (Table 1). The time gap between onset of disease and blood sampling can affect results of blood tests. Therefore, all blood samples from the group of patients without EM were obtained based on the time after onset of symptoms. We found that blood samples from *B. miyamotoi* PCR-positive patients were obtained significantly earlier (z = -3.29, p<0.001, by Mann-Whitney U-test). The median time of blood sampling was 2.0 days (IQR 2.0–4.0 days) for PCR-positive patients without EM and 4.0 days (IQR 2.3–6.0 days) for PCR-negative patients without EM after initial symptoms. Therefore, PCR is significantly lower in patients with and without EM, respectively (z = -3.29, p<0.001, by Mann-Whitney U-test).

![Figure 1. Concentration of *Borrelia miyamotoi* A) RNA and B) DNA in blood samples from patients with *B. miyamotoi* disease during disease progression, Yekaterinburg, Russia, 2009–2010. Blood samples were obtained before antimicrobial drug therapy was given. Boxes indicate interquartile ranges (IQRs), horizontal bars indicate medians, and error bars indicate 1.5× IQR. Circles indicate moderate outliers (1.5×–3× IQR, 238,700 copies/mL), and asterisks indicate extreme outliers (>3× IQR, 280,000–420,000 copies/mL).](863)
Duration of Spirochetemia
In 2010 and 2015, we obtained blood samples repeatedly during the first 6 days of disease from 23 patients with confirmed *B. miyamotoi* disease (Table 2) and used data for these samples to estimate the duration of spirochetemia. We excluded samples from patients who had started antimicrobial drug therapy before or at the same time as the blood tests and those from patients who had a time gap between blood samples >2 days. We performed time-to-event analysis for 17 patients. Pathogen DNA or RNA was detected in blood up to day 5 of illness (Table 2), and all samples showed negative results on day 6 of illness. Median time for detection of pathogen DNA or RNA in blood was 4.0 days (95% CI 3.1–4.9 days) (Figure 3).

Discussion
Borreliosis is the most prevalent tickborne disease in Russia (21,22). Our findings showed that, in Yekaterinburg, ≈23% cases of borreliosis (confirmed clinically, serologically, or by PCR) were caused *B. miyamotoi*. Findings that indicated that *B. miyamotoi* disease might not be a rare infection have been previously suggested for the United States (8,14,23) and the Netherlands (13).

We found that erythema migrans was not apparent in 70 (93%) patients with *B. miyamotoi* disease, and only in 7% of case-patients was erythema migrans manifested. We obtained data for the 5 PCR-confirmed case-patients with *B. miyamotoi* disease and erythema migrans and the case-patient co-infected with *B. burgdorferi* sensu lato and *B. miyamotoi*. We believe that these 5 patients were also co-infected with *Borrelia* spp. and that the *B. burgdorferi* sensu lato DNA was not detected because of the low sensitivity of the PCR. Additional research is needed to test this assumption. Recent studies reported that in the United States, 14% of patients with *B. miyamotoi* disease were co-infected with *B. burgdorferi* (7,23), including a patient with erythema migrans (23).

Our study showed poor sensitivity (≈11%) of qPCR detection for 16S rRNA for *B. burgdorferi* sensu lato.
In comparison, the sensitivity of PCR for *B. burgdorferi* sensu lato varied widely (7.5%–78.1%) (12,24,25). Several studies have reported low sensitivity of PCR for *B. burgdorferi* sensu lato DNA for routine diagnostic purposes because of low numbers of spirochetes circulating in the bloodstream during acute infection (8,12,26–29). For relapsing fever caused by *Borrelia* spp., the number of *glpQ* gene copies for *B. duttonii* and *B. recurrentis* ranged from $10^2$ to $10^4$ copies/mL for 7 (0.3%) of 2,057 healthy participants and from $10^3$ to $10^6$ copies/mL for 15 (3.9%) of 382 patients with fever who were surveyed in Tanzania (16). The number of copies of the *B. miyamotoi* gene in serum samples of 2 patients in Japan was $7.2 \times 10^3$ and $2.8 \times 10^6$ copies/mL by 16S rRNA qPCR (30). A mean copy number of 7,787 copies/mL was reported for *B. miyamotoi* disease patients in the northeastern United States (23).

The number of copies of the *B. miyamotoi* gene in serum samples from 2 patients in Japan was $7.2 \times 10^3$ and $2.8 \times 10^4$ copies/mL by 16S rRNA qPCR (30). A mean copy number of 7,787 copies/mL was reported for *B. miyamotoi* disease patients in the northeastern United States (23). Our study identified the bacterial load in blood samples of patients with *B. miyamotoi* disease ($\leq 9,085$ copies/mL) and showed that an early diagnosis of this disease is possible if a PCR for the 16S rRNA gene is used. The concentration of *B. miyamotoi* RNA in blood is higher than that for DNA. We found a low median RNA:DNA ratio ($\approx 4$), which indicates that use of RNA as the target molecule is inappropriate.

We obtained data on the duration of spirochtemia, which contributes to the early diagnosis of *B. miyamotoi* disease. It is possible to detect borrelial DNA by PCR during the first 3 days of the disease. However, bacterial DNA is then no longer detectable in the blood, so PCR detection is ineffective after 4 days. We detected 73 *Borrelia* spp. ELISA-positive patients without erythema migrans who seroconverted within the observation period and were PCR negative for both *Borrelia* spp. On average, these samples were obtained later than *B. miyamotoi* PCR-positive samples, suggesting that they might have been collected after the end of the period of spirochtemia and caused the PCR-negative results.

We characterized the duration of *B. miyamotoi* spirochtemia during acute illness. Although there was no strong clinical confirmation, our data showed that *B. miyamotoi* DNA and RNA might be detected in the circulation within 30 days after onset of disease. This extended parasitemia is probably related to disease relapse (20). Lee et al. (8) provided indirect results for *B. miyamotoi* spirochtemia and detected *B. miyamotoi* DNA in blood samples of 4 patients during the period with little tick exposure in the northeastern United States. These authors suggested that this off-season spirochtemia with a low bacterial
density was most likely the result of bacteria being dislodged periodically from persistent deep-tissue lesions (8). Thus, a long-term study of pathogen persistence is required.

The duration of B. miyamotoi spirochtemia is relatively short. Thus, the true number of ambulatory and hospitalized patients infected with B. miyamotoi will not be known until a sensitive, reliable, diagnostic laboratory test (i.e., serologic test) is available to detect causative agents in patients with acute infections in disease-endemic areas.

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References


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We demonstrate maintenance and transmission of severe fever with thrombocytopenia syndrome virus by Haemaphy-
salis longicornis ticks in the larva, nymph, and adult stages with dissemination in salivary gland, midgut, and ovarian tis-
sues. The H. longicornis tick is a competent vector to trans-
mitt this virus in both transovarial and transstadial modes.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by SFTS virus (SFTSV), identified in China in 2009 (1) and subsequently in South Korea (2) and Japan (3). Symptoms of SFTS usually include fever, thrombocytopenia, and leukocytopenia; case-fatality rates are 10%–30% (1,4). SFTS is implicated as largely a tick-associated disease, supported by evidence that many patients had exposure to ticks before disease onset (1). The longhorned tick, Haemaphysalis longicornis, the most abundant human-biting tick species in most SFTS-endem-
ic areas of China (5), was found to harbor SFTSV (1,6,7). These studies suggested that H. longicornis ticks might be competent vectors for SFTSV transmission. Our study was designed to determine the role of the H. longicornis tick as a vector in maintenance and transmission of SFTSV.

The Study
We randomly allocated 90 female H. longicornis ticks from an SFTSV-free colony into 2 equal groups, experimental and control. We injected the experimental group with SFTSV and the control group with phosphate-buffered saline (PBS). Seven days postinjection, we used 18 of the 35 live SFTSV-infected ticks for the detection of viral RNA by real-time reverse transcription PCR (rRT-PCR) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/15-1435-Techapp1.pdf); all showed positive results, confirmed by subsequent rRT-PCR and sequenc-
ing analysis. Twelve days postinjection, we dissected 5 live ticks from the experimental group to detect SFTSV in salivary glands and ovaries by indirect fluorescence assay (IFA), which showed notable SFTSV-specific fluorescence (Figure 1, panel A). For the control group, none of the 19 ticks tested by rRT-PCR had SFTSV RNA, and none of the 5 ticks tested by IFA showed SFTSV-specific fluorescence (Figure 1, panel B).

We then let the remaining 12 live ticks from both groups feed on naive Balb/C mice (4 ticks/mouse) until the ticks detached from the mice. The engorged females were harvested and maintained to lay eggs. We deter-
mined transovarial transmission of SFTSV by further testing of SFTSV RNA from eggs, larvae, and nymphs using rRT-PCR. A total of 15 pools of eggs laid by 5 infected H. longicornis ticks (3 pools from each tick, each pool coming from a single female) were SFTSV RNA positive. In contrast, the egg pools from ticks of the control group were all negative. When hatched to larvae, 20 of 25 pools derived from the infected H. longicornis ticks (5 pools from each tick) tested positive for SFTSV RNA; all 25 larvae pools of the control group tested neg-
ative (Table 1).

We further performed transstadial transmission of SFTSV by rearing larvae to adults. All remaining larvae were reared to nymphs and adults by feeding on 20 naive Balb/C mice. We subjected 1 mouse to SFTSV RNA testing after it was bitten by each pool of larvae and the hatched nymphs and adults; we used the other mice for feeding multiple pools of larvae and the hatched nymphs and adults. We fed 3,195 larvae in the experimental group and 2,987 in the control group to engorgement and random-
ly selected and tested engorged larvae. We maintained the other engorged larvae for molting to nymphs. In all, 694 en-
gorged larvae in the experimental group and 652 engorged larvae in the control group successfully molted to nymphs
Transmission of SFTSV by *H. longicornis* Ticks

(online Technical Appendix Tables 1, 2). The remaining 569 nymphs in the SFTSV group and 527 nymphs in the control group were fed on 20 naive Balb/C mice; 453 nymphs in the SFTSV group and 437 in the control group were fully engorged. We divided the remaining engorged nymphs into 5 replicate cohorts; 166 engorged nymphs (39% ± 6% standard error [SE]) in the experimental group and 155 (38% ± 5% SE) in the control group matured to adults (online Technical Appendix Tables 1, 2). The overall hatching rate of eggs and molting rates of nymphs and adults in the 2 groups were comparable.

All 25 engorged larvae pools (5 pools from each mother) in the SFTSV-infected group and none from the control group were positive for SFTSV RNA (Table 1). After the larvae molted to nymphs, 23 of 25 nymph pools from the SFTSV-infected group and none from the control group tested positive for SFTSV RNA. Similarly, all 25 engorged nymph pools from the SFTSV-infected group and none from the control group were positive. When the second generation emerged, we tested 50 adults (25 females, 25 males) in each group for SFTSV RNA; in the SFTSV-infected group, 44% (11/25) of the females and 36% (9/25) of the males tested positive, whereas all 25 females and 25 males in the control group were negative. Positive samples were confirmed by identical sequences to that of the inoculated virus strain.

A total of 83 naive Balb/C mice were infested by ticks (online Technical Appendix Table 3). All 3 Balb/C mice fed by the SFTSV-infected females were positive for exposure to SFTSV 1 week after the ticks detached. Of the naive Balb/C mice that were bitten by larvae from the SFTSV-infected group, 4 of 5 were positive for SFTSV RNA, as were 4 of 5 mice bitten by nymphs, 4 of 5 mice bitten by adult female ticks, and 3 of 5 mice bitten by male ticks; mice bitten by ticks from the control group were negative (Table 2). We used IFA to test serum samples from the mice collected before and 3 weeks after detachment of ticks at different developing stages; all mice positive for SFTSV RNA demonstrated seroconversion against SFTSV (Table 2).

Three of 4 pools of saliva and hemolymph from the experimental group were SFTSV RNA positive. We selected 5 females at random from each group to detect SFTSV in tissues by IFA. The salivary glands, midguts, and ovaries of the SFTSV-injected group displayed SFTSV-specific fluorescence (Figure 2).

![Image A](A) Specific detection of severe fever with thrombocytopenia syndrome virus (SFTSV) in microinjected *Haemaphysalis longicornis* ticks by indirect fluorescence assay. A) SFTSV-injected ticks 12 days after microinjection (original magnification ×10). Red arrows indicate specific fluorescence s. B) Phosphate-buffered saline–injected ticks 12 days after microinjection (original magnification × 10). No specific fluorescence is seen.

![Image B](B)

**Table 1.** Detection of severe fever with thrombocytopenia syndrome virus RNA in experimental and control *Haemaphysalis longicornis* ticks

<table>
<thead>
<tr>
<th>Source</th>
<th>Experimental infection group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>% Positive ± SE</td>
</tr>
<tr>
<td>Mother tick carcasses</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>Egg pool*</td>
<td>15</td>
<td>100</td>
</tr>
<tr>
<td>Larvae pool†</td>
<td>25</td>
<td>80.0 ± 1.7</td>
</tr>
<tr>
<td>Engorged larvae pool‡</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Nymph pool§</td>
<td>25</td>
<td>92.0 ± 1.4</td>
</tr>
<tr>
<td>Engorged nymph</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>Male adult</td>
<td>25</td>
<td>36.0 ± 5.4</td>
</tr>
<tr>
<td>Female adult</td>
<td>25</td>
<td>44.0 ± 2.9</td>
</tr>
<tr>
<td>Female hemolymph pool¶</td>
<td>3</td>
<td>66.7 ± 9.4</td>
</tr>
<tr>
<td>Female saliva pool#</td>
<td>4</td>
<td>75.0 ± 14.4</td>
</tr>
<tr>
<td>Male hemolymph pool¶</td>
<td>3</td>
<td>33.3 ± 9.4</td>
</tr>
</tbody>
</table>

*Eggs were tested in pools of 60.
†Larvae were tested in pools of 50.
‡Engorged larvae were tested in pools of 5.
§Nymphs were tested in pools of 5.
¶Hemolymph collected from 5 ticks was pooled as 1 sample.
#Saliva collected from 5 ticks was pooled as 1 sample.
We observed a significantly higher level of viral load in second-generation eggs than in second-generation adults (p < 0.001 by Mann-Whitney U-test). We also found a significantly higher level (p < 0.0001) of viral load in saliva of engorged second-generation adults than in saliva of unengorged adults, indicating that SFTSV had multiplied.

Conclusions

We report the experimental maintenance and transmission of SFTSV in *H. longicornis* ticks. After microinjection of SFTSV, the virus disseminated in ovaries and salivary glands. Infected *H. longicornis* ticks could transmit SFTSV successfully in both transovarial and transstadial modes. The appearance of SFTSV in saliva and hemolymph suggests that the virus circulates in the tick hemocoel and is expressed in saliva. In addition, naive Balb/C mice infested with experimentally infected adults, larvae, and nymphs all became infected, evidenced by both detection of SFTSV-specific RNA and seroconversion.

These findings, together with data on natural infection in the field (1,6), implicate *H. longicornis* ticks as competent vectors for SFTSV. However, the evidence derived from IFA and rRT-PCR tests could not indicate that the virus is infectious. More efforts should be taken to demonstrate the infectivity of SFTSV in the transmission cycle.

*H. longicornis* ticks are widely distributed in the Asia-Pacific region (8–12). Predominant hosts of *H. longicornis* ticks include humans, poultry, livestock, wild rodents, and birds (12–14). As displayed in mice in the current research, SFTSV is likely to be maintained through vertical and horizontal transmission in ticks that infest these wild and domestic mammals. This maintenance has been evidenced by an extraordinarily high prevalence of SFTSV in sheep, cattle, dogs, pigs, and other animals (7,14). In areas where *H. longicornis* ticks are endemic, infested animals could be considered as key reservoirs in maintaining and transmitting SFTSV (15). The close contact between animals and their owners could pose another way of acquiring infection, in addition to tick bites.

Table 2. Detection of severe fever with thrombocytopenia syndrome virus in *Haemaphysalis longicornis* tick–infested mice*

<table>
<thead>
<tr>
<th>Stage (sex)</th>
<th>No. mice</th>
<th>No. ticks/mouse</th>
<th>No. positive by rRT-PCR</th>
<th>No. positive by IFA</th>
<th>Titer ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adults (female)</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3.01 ± 0.30</td>
</tr>
<tr>
<td>Larvae</td>
<td>5</td>
<td>50</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Nymphs</td>
<td>5</td>
<td>10</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Adults (female)</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Adults (male)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

*IFA, indirect fluorescence assay; rRT-PCR, real-time reverse transcription PCR.

Figure 2. Specific detection of severe fever with thrombocytopenia syndrome virus (SFTSV) in tissues of adult *Haemaphysalis longicornis* ticks by indirect fluorescence assay. The green fluorescence represents the SFTS virus. A) Salivary gland of SFTSV-injected tick (original magnification ×40). B) Midgut of SFTSV-injected *H. longicornis* tick (original magnification ×10). C) Ovary of SFTSV-injected tick (original magnification ×40). D) Salivary gland of phosphate-buffered saline (PBS)–injected tick (original magnification ×40). E) Midgut of PBS-injected *H. longicornis* tick (original magnification ×10). F) Ovary of PBS-injected tick (original magnification ×40).
Transmision of SFTSV by H. longicornis Ticks

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References

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We investigated 1,228 residents of 3 rural areas in South Korea and determined that 50 (4.1%) were positive for severe fever with thrombocytopenia syndrome virus antibodies. Fever and gastrointestinal symptoms in the previous 3 years and career duration were associated with virus seropositivity.

Severe fever with thrombocytopenia syndrome (SFTS) is a tick-transmitted, acute febrile disease caused by SFTS virus (SFTSV) (1,2). Previous studies have not determined the seroprevalence of SFTSV in South Korea. Therefore, we investigated SFTSV seroprevalence among residents of rural areas in South Korea and identified factors associated with seropositivity.

The Study
We conducted our study in rural areas of 3 provinces (Myeoncheon-myeon, Dangjin-gun, Choongcheongnam-do Province; Nodong-myeon, Boseong-gun, Jeollanam-do Province; and Gahoe-myeon, Hapcheon-gun, Gyeongsangnam-do Province) in South Korea that had reported SFTS patients to the Korean Center for Disease Control during 2013–2014 (Figure 1) (3). In September 2014, we administered a structured questionnaire regarding demographic characteristics and occupational and living conditions to 1,228 residents of these areas. We collected blood samples from these persons and subjected them to indirect immunofluorescent assays to determine SFTSV IgG titers. Participants were defined as being seropositive if the indirect immunofluorescent assay IgG titer was >1:32 (Figure 2). Written consent was obtained from all participants before administration of the survey and blood tests. This study was approved by the Chosun University Institutional Review Board.

Of 1,228 persons included in the analysis (Table 1, https://wwwnc.cdc.gov/EID/article/24/5/15-2104-T1.htm), 786 (64.0%) were women, 831 (67.7%) were ≥65 years of age, and 713 (58.1%) worked in agriculture. A total of 225 (18.3%) participants had lived in the same residence for 1–20 years, and 757 (61.6%) had lived in the same residence for ≥41 years; 255 (20.8%) had raised domestic animals and livestock (among which dogs were most common). Furthermore, 166 (13.5%) had received a tick bite during their lifetime (Table 1). The highest tick bite rate was reported in Boseong (94, 26.8%), followed by Hapcheon (43, 10.7%) and Dangjin (29, 7.1%). In the year before the study, 75 (6.1%) of patients in the total cohort had a tick bite (Boseong: 52, 12.5%; Dangjin: 13, 3.2%; Hapcheon: 10, 2.5%), and 25 (2.0%) had a fever and gastrointestinal (GI) symptoms (i.e., SFTS symptoms) during the previous 3 years.

Among the total sample, 50 (4.1%) persons were seropositive for SFTSV (Figure 1): 23 (5.5%) in Boseong, 16 (4.0%) in Hapcheon, and 11 (2.7%) in Dangjin. Antibody seroprevalence was 2.3% (9/397) for persons <64 years of age and 4.9% (41/831) for persons ≥65 years of age (Table 1). Persons who had fever and GI symptoms in the previous 3 years were more likely to be seropositive. Antibody positivity was also higher for persons with a long career duration, but we did not identify any correlations with occupation type, outdoor activity–related characteristics, or type of work in the previous year. Furthermore, 50 persons had antibody titers >1:32, among whom 6 had had a tick bite in the previous year. Among these 6 persons, 5 (83.3%) had an antibody titer >1:128. For 44 persons who did not have tick bites in the previous year, 15 (34.1%) had an antibody titer >1:128. Persons who had a tick bite in the past year had significantly higher antibody titers (p = 0.021).

We used multiple logistic regression analysis to identify variables with significant (p<0.1) probabilities of being associated with seropositivity. Persons who had fever and GI symptoms in the previous 3 years (odds ratio [OR] 4.09, 95% CI 1.25–13.36) and those who had a career duration of ≥41 years (OR 2.36, 95% CI 1.11–5.02) had a higher likelihood of seropositivity than nonsymptomatic persons and those who had a career duration of 1–20 years (Table 2). In addition, of 25 (2%) persons with fever and GI symptoms or suspected SFTS
Symptoms in the previous 3 years, 4 (16.0%) were SFTS seropositive; among the 1,202 with no suspected SFTS symptoms, 3.8% (n = 46) were SFTS seropositive (p = 0.002). Persons who had fever and GI symptoms in the previous 3 years were more likely to be seropositive for SFTSV.

**Conclusions**

A much higher mortality rate was observed for patients with SFTS in South Korea than for patients in China (4). In addition, the high SFTS case-fatality rate in South Korea (47.2%, 17/36) is a serious public health concern (5). The 3 areas examined in this study were rural, and most residents...
were elderly agriculture workers. In China, queried sero-positive farmers denied having typical SFTS symptoms (6). In contrast, despite possible recall bias in our study, seroprevalence was higher for patients who reported fever and GI symptoms (i.e., SFTS symptoms) during the previous 3 years.

When 2,510 residents of Jiangsu Province, China, were subjected to SFTSV antibody testing, 1,104 (0.44%) were seropositive (6). In contrast, a study of 2,547 farmers in a rural area of the same province reported a seropositivity rate of 1.3% (7). Thus, SFTSV seroprevalence was 3-fold higher for rural farmers than for the general population (6, 7). Li et al. also reported that seroprevalence tended to increase with age (7). These findings might help to explain the relatively high seroprevalence (4.1%) observed in our study.

Moreover, studies have indicated that mild or subclinical SFTS might be common. For example, SFTS viral antibody testing of healthy residents in Zhejiang Province, China, showed that 7.2% had IgG against SFTSV (8). In this study, when persons were asked if they had had GI symptoms and fever in the previous 3 years, which indicated a suspected SFTSV infection, 25 (2.0%) persons, including 4 (8.0%) of 50 seropositive persons reported that they had had these suspected SFTS symptoms. Furthermore, persons who reported suspected SFTS symptoms were more likely to be seropositive. Therefore, subclinical or mild SFTSV infections might be present in the study communities.

In addition, the higher antibody titers for persons who had ticks bites in the past year indicate a correlation between tick bites and antibody titer positivity. Long career duration was associated with SFTS seroprevalence in this study. Most study participants were elderly; age and career duration showed a weak positive correlation (r = 0.312, p<0.001). Additional research is needed to investigate SFTSV seroprevalence, including various age groups containing an adequate number of persons.

Our study had some limitations. First, we did not use other seropositivity testing methods, such as ELISA, because of lack of availability. Second, recollection of symptoms (e.g., fever) on the questionnaire might have introduced recall bias.

In summary, SFTSV seroprevalence was 4.1% for residents in 3 rural areas of South Korea. A history of fever and GI symptoms and a long career duration were associated with SFTSV seroprevalence.

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References

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Human Usutu Virus Infection with Atypical Neurologic Presentation, Montpellier, France, 2016

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Infection with Usutu virus (USUV) has been recently associated with neurologic disorders, such as encephalitis or meningoencephalitis, in humans. These findings indicate that USUV is a potential health threat. We report an acute human infection with USUV in France putatively associated with a clinical diagnosis of idiopathic facial paralysis.

Usutu virus (USUV) is a mosquitoborne flavivirus. This virus was detected in South Africa in 1959 and is maintained through an enzootic cycle involving birds as the main amplifying reservoir hosts and ornithophilic mosquito species as vectors (mainly Culex spp.) (1). Mammals, including humans, are incidental dead-end hosts. Although USUV was considered a tropical or subtropical virus, it was recently introduced in central and western Europe. Emergence of USUV in Europe was reported in Austria in 2001, but retrospective analyses have suggested an earlier introduction because several epizootics and small outbreaks among local birds have been suspected since 1996 (1). In 2016, a large USUV epizootic was reported in Belgium, France, Germany, and the Netherlands (2).

The zoonotic potential of USUV was initially described in the Central African Republic and recently confirmed in Europe by reports of neuroinvasive infections caused by this virus (1). Further evidence of probable human infections was demonstrated by sero-prevalence studies on healthy blood donor samples: prevalence of 1.1% in Italy (3) and 0.02% in Germany (4). Furthermore, a recent blood donor screening in Germany identified an acute USUV infection (5). Although human infections have not been identified in France, deaths of birds during 2015–2016 confirmed USUV circulation (6,7).

Moreover, recent data have shown a high prevalence (7%) of USUV in Culex pipiens mosquitoes in the Rhone River delta, a region also called Camargue, in 2015 (M. Eiden et al., unpub. data). Camargue is a landscape of wetlands that hosts a diversity of wild bird species, including migratory birds, and diverse mosquito populations. This environment could potentially favor transmission of USUV to humans, similar to that for West Nile virus (WMV) in this area (8).

We investigated the zoonotic potential of USUVs and WNVs in France by a retrospective flavivirus molecular survey of cerebrospinal fluid (CSF) samples collected in 2016 during the period of maximum mosquito activity (May–November). Samples were obtained from patients with infectious or neurologic syndromes in 2 towns near Camargue. One CSF sample was positive for USUV RNA. We report detection of an acute human infection with USUV in France associated with an unexpected clinical diagnosis of idiopathic facial paralysis.

The Study
We retrospectively screened a collection of RNA extracts stored at –80°C by using a modified consensual panflaviruses assay (9) with a One-Step RT-PCR Sybr-Green Mixture (Qiagen, Hilden, Germany). Extracts were obtained from 666 CSF samples collected at the Université de Montpellier Hôpital (Montpellier, France) and Nîmes University Hospital (Nîmes, France) during May–November 2016. Samples are part of a registered systematic collection established for epidemiologic purposes during the surveillance period for risk of infection with arboviruses (May–November) in a region of southern France that has Aedes albopictus mosquito vectors (Table). RNA extracts from samples with a previous probable bacterial or viral etiology were not assessed.

One sample showed a positive reverse transcription PCR (RT-PCR) result for panflaviruses (cycle threshold 33). Subsequent Sanger dideoxy sequencing with amplification primers for a 260-bp nonstructural protein 5 gene

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sequence identified a USUV RNA sequence. Phylogenetic analysis based on this partial sequence, previously shown to accurately discriminate USUV lineages (2,10), identified a strain closely related to viruses that were circulating in birds in southern Europe (Figure 1).

We tested a remaining stored aliquot of a CSF sample for USUV by using a specific USUV RT-PCR (cycle threshold 30) (11). We inoculated this aliquot onto Vero cells and primary human astrocytes (12) after a round of amplification in C6/36 cells. Infected Vero cells showed a typical cytopathic effect (Figure 2, panel A) (13). We also detected infected astrocytes by immunofluorescence, thus demonstrating the presence of infectious viral particles

Table. Clinical conditions or symptoms associated with microbial investigations of cerebrospinal fluid samples for infections with arboviruses, France*

<table>
<thead>
<tr>
<th>Condition</th>
<th>No. (%) positive samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meningitis/encephalitis</td>
<td>277 (41.6)</td>
</tr>
<tr>
<td>Neurologic disorders†</td>
<td>233 (34.9)</td>
</tr>
<tr>
<td>Febrile syndrome</td>
<td>108 (16.2)</td>
</tr>
<tr>
<td>Other</td>
<td>48 (7.0)</td>
</tr>
<tr>
<td>Total</td>
<td>666 (100.0)</td>
</tr>
</tbody>
</table>

*Samples collected at the Université de Montpellier Hôpital (Montpellier, France) and Nîmes University Hospital (Nîmes, France) during May–November 2016.†Including (>2%) convulsion/epileptic seizure (25%); paralysis/paresthesia/polyradiculoneuritis/motor loss/palsy (31%); myelitis (2%); vascularitis (3%); encephalopathy (6%); Guillain-Barré syndrome (4%); headache (15%); and confusion (10%).

Figure 1. Phylogenetic relationship of the France-Montpellier-2016 strain of Usutu virus (USUV) (box; GenBank accession no. LT854220), isolated from a 39-year-old man in Montpellier, France, who had an atypical neurologic presentation, compared with other USUV strains based on the partial nonstructural protein 5 gene sequence. USUV sequences are shown with their country of isolation, year of isolation, and GenBank accession numbers. Hosts from which the strains were detected (bird, mosquito, bat, or human) are shown next to strain names. Analysis was processed through the French phylogeny website (http://www.phylogeny.fr). Nucleotide sequences were aligned by using MUSCLE software (https://www.ebi.ac.uk/Tools/msa/muscle/). The phylogenetic tree was constructed by using the maximum-likelihood method in PhyML (http://www.atgc-montpellier.fr/phyml/). One hundred bootstrap datasets with random sequence addition were computed to generate a consensus tree drawn with TreeDyn software (http://www.treedyn.org/) and rooted with a West Nile virus sequence (GenBank accession no. AF196835). Numbers along branches are bootstrap values. Map shows locations where USUV strains were detected in France during 2015–2016. Scale bar indicates nucleotide substitutions per site.
(Figure 2, panel B). However, we detected no viremia in a blood sample by specific USUV RT-PCR and no specific USUV antibodies. Although virus and antibody dynamics are unknown for acute USUV infection, it is likely that these samples, collected 3 days after onset of symptoms, were collected too early to detect antibodies, as observed for other flaviviruses (14).

The case-patient was a 39-year-old man admitted to the Department of Neurology, Université de Montpellier Hôpital on November 10, 2016, because of sudden peripheral facial palsy and eyelid ptosis after prodromal dysgeusia. We obtained informed consent from the case-patient to publish his results.

The patient did not report any previous infectious signs or history of recent travel. Symptoms onset occurred 3 days before admission, and at the time of admission, he experienced gradual paresthesias of both right limbs and reported transient right upper limb palsy for ≈1 h. No other objective alteration of cranial nerves was detected during neurologic examination. Sensitivity was normal with presence of regular osteo-tendinous reflexes. Blood cell count, levels of liver enzymes, and renal function results were within reference ranges. No inflammatory or infectious syndromes were observed.

C-reactive protein level in a blood sample was 0.6 mg/L (reference value <5 mg/L). CSF protein level was 67 mg/dL (reference range 15–35 mg/dL). There was no pleiocytosis. Results of CSF cultures and routine PCRs were negative for herpes simplex virus, varicella zoster virus, and enteroviruses. Magnetic resonance imaging of the brain showed unremarkable results: no ischemic disorders on diffusion-weighted imaging and no enhancement with gadolinium in brainstem and cranial nerves. Results of nerve conduction studies on the 4 limbs were normal. The patient was given a diagnosis of idiopathic facial paralysis.

The patient was given corticoids, valaciclovir, and eye drops for prevention of keratitis. He was discharged 3 days later, and symptoms of facial palsy disappeared within a few weeks.
Conclusions
Since the first major epizootic event in 2002, continuous geographic expansion of the range of USUV in Europe has been shown by reports of epizootics or small outbreaks from various countries in western Europe, with widespread activity of multiple lineages (2). Thus, USUV has likely become a potential human health concern and an increasing number of human infections have been described. Our report suggests an acute human USUV infection and shows that circulation of USUV involves a wider geographic distribution in France than reported (2). Phylogenetic analysis of the virus sequence isolated from the patient showed a strain probably related to the USUV/Spain strain, which has been detected in common blackbirds in France (6). Because our study region was near Camargue, detection of similar strains upstream and at the mouth of the Rhone River is consistent.

The zoonotic potential of USUV infection in Europe has been underestimated in a limited number of cases, including reports of encephalitis or asymptomatic cases (1). However, a retrospective study in a disease-endemic area in Italy showed that human USUV infection is not a sporadic event and showed a higher incidence than infection with WNV (7). For our case-patient, absence of evidence of an infectious syndrome associated with a clinical neuromuscular presentation of acute unilateral facial paralysis is atypical. However, because idiopathic facial paralysis, also known as Bell’s palsy, could be caused by ischemic, immune, and infective mechanisms (15) and the well-described neurotropism of some flaviviruses, the etiologic role of USUV must not be ruled out.

Our report of an acute human USUV infection in France reinforces the need for integrated surveillance in animals, vectors, and humans. The atypical clinical presentation remains an intriguing point that deserves more investigations and suggests that human USUV infections might display various clinical patterns and could have been underestimated.

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Alkhurma Hemorrhagic Fever Virus RNA in Hyalomma rufipes Ticks Infesting Migratory Birds, Europe and Asia Minor

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Alkhurma hemorrhagic fever virus RNA was detected in immature *Hyalomma rufipes* ticks infesting northward migratory birds caught in the North Mediterranean Basin. This finding suggests a role for birds in the ecology of the Alkhurma hemorrhagic fever virus and a potential mechanism for dissemination to novel regions. Increased surveillance is warranted.

Alkhurma hemorrhagic fever virus (AHFV) was identified in 1995 after an outbreak of viral hemorrhagic fever in Jeddah Province, Saudi Arabia (1). This virus is a variant of Kyasanur Forest disease virus (KFDV), which is endemic in eastern India, and a member of the mammalian tickborne flaviviruses (2). An association between Alkhurma hemorrhagic fever cases and livestock handling was identified early on, suggesting goats and sheep are potential reservoirs (3). Furthermore, AHFV has been identified in *Ornithodoros savignyi* soft ticks and *Hyalomma dromedarii* hard ticks (potential vectors) in Saudi Arabia, both of which are associated with camels (potential reservoir) (4,5). Alkhurma hemorrhagic fever is endemic in several provinces of Saudi Arabia; sporadic cases have been reported in Africa near the Egypt–Sudan border and cases of seropositivity in Djibouti (6–8) (Figure 1). AHFV RNA has also been detected in *Amblyomma lepidum* ticks collected from cattle in Djibouti (9).

The clinical manifestation of Alkhurma hemorrhagic fever resembles that of other viral hemorrhagic fevers: initial malaise and influenza-like symptoms, followed by encephalitis, icterus, and ecchymosis. Case fatality is ≈25% but could be considerably lower, considering mild cases are probably undiagnosed (1). It has been suggested that both KFDV and AHFV originated in Africa and that, subsequently, KFDV spread to India and AHFV to Saudi Arabia, KFDV possibly disseminating farther to southern China by migratory birds (2). Also, other pathogens have been found to disseminate by means of ticks on migratory birds (10). In light of these findings, case reports in Africa (6–8), and increasing case frequency in Saudi Arabia (11), we investigated whether ticks infesting migratory birds en route from Africa to Europe and Asia during springtime carry AHFV.

The Study

We collected ticks from birds migrating northward initially at 2 bird observatories on the Mediterranean islands Capri (Italy; 40°33′N, 14°15′E) and Andikithira (Greece; 35°51′N, 23°18′E) during spring of 2009 and 2010. We captured 14,824 birds (78 species) during their yearly migration, presumably leaving Africa for breeding grounds in Europe or Asia. We collected 747 ticks, 88% of which were identified morphologically as members of the complex *H. marginatum* sensu lato (s.l.), most likely *H. rufipes* and *H. marginatum*. We screened cDNA for AHFV by real-time PCR with primers targeting the 5′ untranslated region of AHFV (12). Five fully engorged ticks, morphologically and molecularly determined to be *H. marginatum* s.l., likely *H. rufipes* (GenBank accession nos. MH061004–MH061008, MH061010–MH061014), tested positive for AHFV RNA in 2 separate analyses. These 5 ticks (4 nymphs, 1 larva) were collected at Andikithira in 2010 (Table) from 3 bird species that winter in sub-Saharan Africa and breed in Europe (Figure 1, panels A–C). One sedge warbler (*Acrocephalus schoenobaenus*) carried 2 AHFV-positive nymphs. Sequencing of the 5′ untranslated region amplicons revealed 2 identical
72-bp sequences. BLAST (http://www.ncbi.nlm.nih.gov/blast/) and comparative analyses revealed high identity to AHFV and KFDV reference sequences, despite a region with 7 consecutive mismatching nucleotides and a 14-nt deletion (Figure 2, panel A). Apart from this difference, 100% identity was seen with all other AHFV and KFDV sequences available in GenBank.

Because of these results, we included additional bird observatories and collection sites along with Capri and Andrikíthira in spring of 2014 and 2015: the Anapodaris River, Crete, Greece (34°59′N, 25°17′E); Huelva Province, Spain (37°30′N, 5°30′W); Sevilla Province, Spain (37°33′N, 6°55′W); Canary Islands, Spain (28°9′N, 15°25′W); Jerusalem, Israel (31°47′N, 35°13′E); and the Kizilirmak Delta, Turkey (41°38′N, 36°05′E). We ringed 22,069 birds (137 species) and collected 1,024 ticks. RNA was extracted at Uppsala University (Uppsala, Sweden) and sent to Agence Nationale de Sécurité Sanitaire de l’Alimentation (Paris, France) for high-throughput screening by microfluidic real-time PCR (Biomark Dynamic Arrays, Fluidigm, South San Francisco, CA, USA) targeting multiple tickborne viruses (S. Moutailler, unpub. data). One fully engorged adult tick, molecularly determined to be *H. marginatum* s.l. (GenBank accession nos. MH061009, MH061015), likely *H. rufipes*, collected from a common redstart (*Phoenicurus phoenicurus*) in Turkey tested positive for AHFV RNA (Table) when using a set of primers and probe that amplifies part of the KFDV/AHFV premembrane region (Kyasanur_poly_F, 5′-ACACGATGCACACACCTGC-3′; Kyasanur_poly_R, 5′-CACCAATGAAACTCTAGTCGTC-3′; Kyasanur_poly_P, 5′-AGAACCGGGACTTTGTCTCAGGGAC-3′).

To confirm this finding, a subsequent real-time PCR was

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**Table.** Characteristics of birds infested by ticks testing positive for Alkhurma hemorrhagic fever virus RNA by real-time PCR, Greece and Turkey, 2010 and 2014

<table>
<thead>
<tr>
<th>Ring no.</th>
<th>Bird species</th>
<th>Capture date</th>
<th>Tick species</th>
<th>Life stage</th>
<th>Capture site</th>
</tr>
</thead>
<tbody>
<tr>
<td>B913855</td>
<td>Eastern woodchat shrike (<em>Lanius senator niloticus</em>)</td>
<td>2010 May 5</td>
<td><em>Hyalomma marginatum sensu lato</em> (H. <em>rufipes</em>)</td>
<td>Nymph</td>
<td>Greece</td>
</tr>
<tr>
<td>A228919</td>
<td>Sedge warbler (<em>Acrocephalus schoenobaenus</em>)</td>
<td>2010 May 7</td>
<td><em>H. marginatum</em> s.l. (H. <em>rufipes</em>)</td>
<td>Nymph</td>
<td>Greece</td>
</tr>
<tr>
<td>A226883</td>
<td>Western yellow wagtail (<em>Motacilla flava</em>)</td>
<td>2010 May 9</td>
<td><em>H. marginatum</em> s.l. (H. <em>rufipes</em>)</td>
<td>Larva</td>
<td>Greece</td>
</tr>
<tr>
<td>A225166</td>
<td>Sedge warbler (<em>A. schoenobaenus</em>)</td>
<td>2010 May 13</td>
<td><em>H. marginatum</em> s.l. (H. <em>rufipes</em>)</td>
<td>Nymph</td>
<td>Greece</td>
</tr>
<tr>
<td>A225166</td>
<td>Sedge warbler (<em>A. schoenobaenus</em>)</td>
<td>2010 May 13</td>
<td><em>H. marginatum</em> s.l. (H. <em>rufipes</em>)</td>
<td>Nymph</td>
<td>Greece</td>
</tr>
<tr>
<td>JB53791</td>
<td>Common redstart (<em>Phoenicurus phoenicurus</em>)</td>
<td>2014 Apr 29</td>
<td><em>H. marginatum</em> s.l. (H. <em>rufipes</em>)</td>
<td>Adult</td>
<td>Turkey</td>
</tr>
</tbody>
</table>

---

**Figure 1.** Wintering (light brown) and breeding (green) locations and springtime migratory routes (arrows) of birds testing positive for Alkhurma hemorrhagic fever virus (AHFV) RNA in Greece and Turkey, 2010 and 2014. The 4 bird species found infested by *Hyalomma* ticks carrying AHFV RNA were the western yellow wagtail (*Motacilla flava*) (A), eastern woodchat shrike (*Lanius senator niloticus*) (B), sedge warbler (*Acrocephalus schoenobaenus*) (C), and common redstart (*Phoenicurus phoenicurus*) (D). Red shading indicates areas where AHFV has been detected. The dashed line shows the approximate northern geographic boundary of *H. marginatum* complex ticks (based on information from the European Center for Disease Prevention and Control, https://ecdc.europa.eu/en/disease-vectors/surveillance-and-disease-data/tick-maps). *H. rufipes* ticks have a wide geographic distribution in Africa and are present in Saudi Arabia. Collection sites are labeled: Andikíthira, Greece (A); Capri, Italy (C); Jerusalem, Israel (J); Kizilirmak Delta, Turkey (K); Huelva and Sevilla Provinces, Spain (S); Canary Islands, Spain (L); and Crete, Greece (T). Maps created based on information from The Birds of the Western Palearctic, volumes 5–7, Oxford (UK): Oxford University Press; 1988, 1992, 1993.
performed with the same primers and probe. Alignment of the 31-bp fragment revealed a single nucleotide change compared with available AHFV sequences and 3 or 4 differences compared with available KFDV sequences (Figure 2, panel B). The methods used did not enable virus isolation and propagation; therefore, acquisition of further sequence information proved difficult. The common redstart also breeds in Europe and winters in sub-Saharan Africa, similarly to the 3 previously mentioned bird species (Figure 1, panel D).

Conclusions

We detected AHFV/KFDV RNA in 6 of 1,771 investigated ticks. Although we could not differentiate between KFDV and AHFV on the basis of the short sequences analyzed, we suggest these sequences represent AHFV because this supposition is geographically and ecologically more plausible. Our findings are insufficient to establish the role of the *H. rufipes* tick as an AHFV vector. However, detection of AHFV RNA in Europe and Asia Minor raises concerns for conceivable dissemination of the virus to these areas, facilitated by climate change resulting in increased distribution of *Hyalomma* spp. ticks. *H. rufipes* ticks are widely distributed in Africa and are also present on the Arabian Peninsula, along the Red Sea coast (13). The *H. rufipes* species is a 2-host tick; that is, the nymph remains attached to ingest blood on the same host it did for its first blood meal as a larva. Immature ticks usually infest birds or small mammals (e.g., hares), whereas adult ticks feed on larger mammals (e.g., camels, buffaloes, cattle, and large birds) (13). Consequently, the AHFV-positive ticks (except the 1 adult) most likely had fed only on the bird to which they were attached. This assumption implies that an immature tick could have acquired the virus vertically from its mother, from its putatively viremic avian host, or by co-feeding transmission, which can occur when ticks feed close to ≥1 infective tick (14,15). However, in this case, co-feeding transmission is less plausible because the 2 ticks attached to the same bird were not located close to each other (on the beak and nape). For several tickborne viruses, including KFDV, vertical transmission from the adult female tick to the eggs occurs. However, the prevalence of transovarial infection in nature is considered low (14). The 1 positive adult tick could have acquired virus in any of these ways or from a previous mammalian blood meal.

To our knowledge, birds have not been shown to play a role in the ecology or epidemiology of AHFV, and this tick species has not been identified previously as a possible vector. Our finding of this virus in ticks infesting birds en route from Africa to Europe and Asia, together with clinical cases in southern Egypt, the detection of AHFV RNA in ticks, and cases of seropositivity in Djibouti, could indicate a wider geographic distribution of the virus throughout eastern Africa and in novel regions. Further investigations of AHFV ecology and modes of transmission, including the potential role of *Hyalomma* ticks as vectors and passerine birds as reservoirs or distributors of potentially infected ticks, as well as increased surveillance, are needed.

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We thank Dario Piacentini, Laura Gangoso, Rafael Gutierrez, Manuel Vazquez, Jose Luis Arroyo, Alberto Pastoriza, and all others involved in the tick collections and Peter Wilhelmsson for sample organization.

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References


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Combining the official cholera line list data and outbreak investigation reports from the ministries of health in Uganda and South Sudan with molecular analysis of *Vibrio cholerae* strains revealed the interrelatedness of the epidemics in both countries in 2014. These results highlight the need for collaboration to control cross-border outbreaks.

Most countries in sub-Saharan Africa are affected by cholera epidemics ranging from annually to every 3–5 years or more (1,2). Cholera tends to be reported at the national or subnational level with few attempts to understand how it may simultaneously affect multiple countries in the same region (3–5). Published studies combining both microbiological and epidemiologic evidence are also scarce. However, better understanding of the interrelatedness of cholera spread between neighboring countries can provide the impetus for more cross-border collaboration in the fight against the disease. This subject is especially relevant in areas with porous borders that experience large population movements, like the border of South Sudan and Uganda.

Large urban communities or cities in cholera-prone areas may play a role in the persistence and transmission of cholera within Africa, given the high volume of travel between cities and other areas and the relatively high population density. Epidemiologic data do show support for the notion that large cities in Africa are hubs of transmission (6), but in-depth analyses are needed to substantiate or refute these hypotheses. Here we explore epidemiologic and microbiological data from cholera epidemics in Uganda and South Sudan in 2014 to establish possible interrelatedness.

The Study
We used the official cholera dataset and outbreak investigation reports from the ministries of health in Uganda and South Sudan. Both countries use the Integrated Disease Surveillance and Response System (https://www.cdc.gov/globalhealth/healthprotection/idsr/about.html) and have adopted similar case definitions from the World Health Organization for areas with confirmed transmission. A suspected cholera case was defined as acute watery diarrhea in a person >2 years of age. A confirmed case was defined as a suspected case in which a stool sample had a culture-positive result for *Vibrio cholerae* O1 or O139.

Cholera case reporting began on April 25, 2014, in Moyo District in northern Uganda, bordering with Kajo-Keji County in South Sudan (Figure 1, panel A). This region reported 88 cases and 3 deaths in the subsequent weeks (Figure 2). The epidemic was contained after rapid implementation of control measures.

Cholera case reporting began on April 29, 2014, in Juba, South Sudan (300 km from Moyo town, Uganda), with a case investigation finding no evidence of travel outside of Juba. Within days, the first reported cholera outbreak in South Sudan since 2009 began, resulting in 6,269 suspected cases, including 105 deaths in health facilities and 51 community deaths (case-fatality ratio 2.4%) by the end of October 2014 (7). Transmission continued in Juba throughout the epidemic, and outbreaks occurred throughout the country, including large outbreaks in the north.

In early July 2014, months after the last confirmed case in Uganda but during a period of intense transmission in South Sudan, a new outbreak was reported in Moyo district, in subcounties (Metu and Dufile) that were not...
affected during the first outbreak (Itirikwa, Aliba, and Gimara); cases were eventually reported in the neighboring Arua district. An investigation revealed that the presumed index case-patient of this second outbreak had traveled to South Sudan. In total, 86 cases and 4 deaths were reported in Moyo and Arua Districts by October 22.

Although not all suspected cases were confirmed during the outbreaks, both countries routinely sent samples to their respective national reference laboratory for microbiological confirmation. We characterized 56 strains at Institut Pasteur (Paris, France) by determining the antimicrobial drug resistance patterns using the disk diffusion method following CA-SFM (Comité de l’Antibiogramme de la Société Française de Microbiologie) 2013 standards for Enterobacteriaceae (http://www.sfm-microbiologie.org/); subtyping with pulse-field gel electrophoresis (8) with SfiI and NorI restriction enzymes (Roche Molecular Biochemicals, Indianapolis, IN, USA); and multilocus variable number tandem repeat (MLVA) analysis targeting 6 loci in the V. cholerae genome (9), and by genotyping tests (10). We used BioNumerics version 6.6 (Applied Maths, Inc., Austin, TX, USA) for clustering analysis (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/17-1651-Techapp1.pdf).

All 56 isolates were V. cholerae O1 serotype Inaba, atypical El Tor biotype, based on rstR^ET, tcp^ET, and ctxB^Cl allele sequences (classical ctxB1 allele) (10). The isolates shared similar antimicrobial drug resistance patterns and were resistant to trimethoprim/sulfamethoxazole, sulfonamides, streptomycin, and nalidixic acid (confirmed by MIC determination [MICs 16–256 mg/L] with Etest; AB bioMérieux, Solna, Sweden). Sequencing of the genes encoding DNA gyrase (gyrA and gyrB) and topoisomerase IV (parC and parE) detected 1 mutation in gyrA (substitution of serine by isoleucine at position 83), which has been associated with quinolone resistance in clinical V. cholerae.

Figure 1. Locations and molecular analysis of 2014 epidemic in Uganda and South Sudan. A) Affected areas in both countries. Light brown indicates districts where we did not obtain any isolate for molecular analysis; red, orange, and blue areas represent affected districts with cholera isolates included in the analysis. B) Multilocus variable-number tandem-repeat (MLVA) analysis. Minimum spanning tree using pairwise difference was generated using BioNumerics version 6.6 (Applied Maths, Inc., Austin, TX, USA). Circles represent the 8 distinct MLVA profiles we identified, numbered chronologically by the earliest isolate of each profile. The MLVA profiles differ by variations at a single variable-number tandem-repeat locus located in the small chromosome only (VCA 0171, VCA 0283). The size of the circles is proportional to the number of isolates in each profile. Colors represent the location of the isolates and correspond with the colors of the areas in Panel A.
isolates (11). PFGE analyses revealed a single SfiI profile and 2 NotI profiles, 1 represented by a single strain. We identified 8 highly related MLVA profiles (Figure 1, panel B) and found variability only in 2 loci on the small chromosome (VCA0171 and VCA0283). All MLVA profiles formed a single clonal complex, in which all isolates can be connected through mutations at a single locus. These results demonstrate genetic uniformity of isolates and provide strong evidence that these epidemics in 2 countries resulted from the spread of a single clone with probable epidemiologic links.

This analysis has several limitations. The identified index cases in each country may not truly have been the initial case-patients, given that the surveillance systems in both locations are not highly sensitive. We selected the cases for culture by convenience sampling. Whereas random sampling is ideal, it is difficult to implement during epidemics because of competing priorities. Furthermore, although studies have shown that 6-locus MLVA can be highly discriminative for identifying a closely related pandemic strain isolated in a small timeframe and geographic area (12,13), our lack of knowledge of MLVA limitations makes it more difficult to draw inferences about strain relatedness and phylogenetic history, especially compared with whole-genome sequencing (14).

Conclusions
Through epidemiologic and molecular data, we illustrated that the 2 outbreaks in South Sudan and Uganda in 2014 clustered into a single epidemic. The spread of cholera from border communities in Uganda to South Sudan and from South Sudan back to Uganda is a critical issue that needs further clarification to improve control strategies. Isolating the neighboring communities from one another is not possible; however, we recommend coordinated interventions by the 2 countries to identify the sources of infection, as was done during Ebola outbreaks in West Africa during 2014–2016.

Figure 2. Epidemic curve of suspected cholera cases in South Sudan (A) and Uganda (B) in 2014, by hospital admission date and region.
The 2014 cholera epidemics probably evolved from a local outbreak in northern Uganda to a national outbreak in South Sudan; population movement, living conditions, and events in the capital, Juba, most likely played a key role in the spread of the disease to other areas in South Sudan and beyond. Refining our understanding of cholera beyond administrative boundaries, perhaps adopting regional approaches in addition to national cholera control efforts, and including key hubs of transmission, such as cities, may be key to minimizing the spatial extent and magnitude of future epidemics.

Joint implementation of disease control interventions and rapid information sharing platforms can strengthen collaboration between states to control the outbreaks. Further studies to describe the relatedness and routes of transmission of *V. cholerae* organisms and track the progression of the outbreaks, combining traditional and molecular epidemiologic tools, can aid public health decision making in Africa and beyond (15). International agencies should facilitate funding and support for joint country activities to expedite control of cross-border cholera epidemics.

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### References


### Table. Characterization of Vibrio cholerae O1 isolates from South Sudan and Uganda, 2014∗

<table>
<thead>
<tr>
<th>Location</th>
<th>No. isolates*</th>
<th>Sample collection period</th>
<th>Location</th>
<th>No. isolates*</th>
<th>Sample collection period</th>
<th>MLVA profile no.</th>
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<th>VC 0437</th>
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<td>2014 June</td>
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<td>9</td>
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*MLVA, multilocus variable-number tandem-repeat analysis; PFGE, pulsed-field gel electrophoresis; VNTR, variable-number tandem-repeat. †PFGE profile number obtained after restriction with NotI and SfiI respectively (NotI/SfiI). The number in parentheses refers to the number of isolates sharing the same profile.


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May 2015: Vectorborne Infections

- Detecting Spread of Avian Influenza A(H7N9) Virus Beyond China
- Recent US Case of Variant Creutzfeldt-Jakob Disease—Global Implications
- Novel Thogotovirus Associated with Febrile Illness and Death, United States, 2014
- Transmission of Hepatitis C Virus among Prisoners, Australia, 2005–2012
- Pathologic Changes in Wild Birds Infected with Highly Pathogenic Avian Influenza (H5N8) Viruses, South Korea, 2014
- Itaya Virus, a Novel Orthobunyavirus Associated with Human Febrile Illness, Peru
- Isolation of *Onchocerca lupi* in Dogs and Black Flies, California, USA
- Molecular Epidemiology of *Plasmodium falciparum* Malaria Outbreak, Tumbes, Peru, 2010–2012
- Delayed-Onset Hemolytic Anemia in Patients with Travel-Associated Severe Malaria Treated with Artesunate, France, 2011–2013
- Protective Antibodies against Placental Malaria and Poor Outcomes during Pregnancy, Benin
- Canine Distemper in Endangered Ethiopian Wolves
- Comparative Sequence Analyses of La Crosse Virus Strain Isolated from Patient with Fatal Encephalitis, Tennessee, USA
- Low-level Circulation of Enterovirus D68–Associated Acute Respiratory Infections, Germany, 2014
- Transmission Potential of Influenza A(H7N9) Virus, China, 2013–2014
- Rapid Emergence of Highly Pathogenic Avian Influenza Subtypes from a Subtype H5N1 Hemagglutinin Variant
- Antimicrobial Drug Resistance of *Vibrio cholerae*, Democratic Republic of the Congo
- Postmortem Stability of Ebola Virus
- Influenza A(H5N8) Virus Similar to Strain in Korea Causing Highly Pathogenic Avian Influenza in Germany
- Malaria Imported from Ghana by Returning Gold Miners, China, 2013
- Canine Infections with *Onchocerca lupi* Nematodes, United States, 2011–2014
- Characterization of *Shigella sonnei* Isolate Carrying Shiga Toxin 2–Producing Gene
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- Ciprofloxacin-Resistant *Shigella sonnei* Associated with Travel to India

https://wwwnc.cdc.gov/eid/content/21/5/contents.htm
We conducted an external quality assessment of Zika virus molecular diagnostic tests in Brazil using a new Zika virus standard. Of 15 laboratories, 73% showed limited sensitivity and specificity. Viral load estimates varied significantly. Continuous quality assurance is required for adequate estimates of Zika virus–associated disease and determination of patient care.

The catastrophic Zika virus outbreak in the Americas has affected millions of persons. Brazil was the most affected country and reported ≈95% of all cases of suspected Zika virus–associated congenital disease (1). Limited sensitivity and specificity of tests hampers serologic detection of Zika virus–specific antibodies in tropical regions (2). Thus, real-time reverse transcription PCR (RT-PCR) has been key for diagnosing acute Zika virus infection and for use in epidemiologic studies (3–5). However, Zika virus molecular diagnostic testing is challenged by short-term viremia and low viral loads (3).

A recent external quality assessment (EQA) in Europe revealed that 60% of laboratories need to improve molecular Zika virus detection (6). Laboratories in affluent countries conduct Zika virus diagnostic testing predominantly in travelers returning from tropical regions. In resource-limited settings to which multiple co-circulating arboviruses are endemic, the diagnostic demands differ entirely. To evaluate the diagnostic landscape in the region most affected by Zika virus, we performed an EQA of molecular Zika virus diagnostic testing in Brazil during 2017.

The Study
Fifteen laboratories from 7 Brazilian states participated in this study; these laboratories are spread across ≈2,500 km longitude, including the areas most affected during Brazil’s Zika virus outbreak (1). Participants were university laboratories, hospital laboratories, federal research institutes supporting public health services, and a diagnostic testing company. We provided EQA panels to all laboratories. Each panel comprised 12 lyophilized samples containing inactivated full virus spiked into human plasma tested negative for arboviruses beforehand. The panel consisted of 4 Zika virus–positive specimens of 10^3–10^6 RNA copies/mL to assess sensitivity and determine viral load. Zika virus–negative specimens to assess specificity comprised dengue virus serotypes 2 and 4, Japanese encephalitis

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virus, St. Louis encephalitis virus, West Nile virus, yellow fever virus, and chikungunya virus at $\approx 10^5$ 50% tissue culture infective dose/mL each and a negative plasma specimen (Table 1). Moreover, each panel included the international World Health Organization (WHO) Zika virus standard for quantification (7). However, the WHO standard has limited availability. Importation of the WHO standard may be restricted by countries that perceive heat-inactivated materials that derive from live virus as potentially hazardous that may be problematic given that viral loads of $10^3$–$10^4$ copies/mL are commonly observed in Zika virus–infected patients (3).

We asked all laboratories to conduct molecular Zika virus diagnostics as routinely done with clinical samples and to quantify Zika virus–positive specimens using both standards. All but 1 laboratory used the same real-time RT-PCR protocol developed by Lanciotti et al. (2), highlighting the wide dissemination of this assay in Brazil and suggesting comparability of test results within this study (Table 1). We found no significant difference between samples containing comparable quantities of the Asian and the African Zika virus lineage, suggesting suitability of the protocols for both lineages ($p = 0.313$ by Fisher exact test).

EQA results varied among laboratories. Of 15 laboratories, 4 (27%) reported correct results for all samples. Five (33%) reported 1 or 2 false-negative results from samples with low Zika virus concentrations (Table 1; Figure 1, panel A). EQA participants correctly tested only the 2 samples containing the highest Zika virus concentrations of $8.1 \times 10^4$ and $7.0 \times 10^4$ copies/mL (exact test of goodness-of-fit $p = 1.00$ and $p = 0.14$, respectively). This finding suggests a potential lack of sensitivity that may be problematic given that viral loads of $10^3$–$10^4$ copies/mL are commonly observed in Zika virus–infected patients (3).

Six (40%) laboratories reported ≥3 false results, including at least 2 false-positive detections of Zika virus–negative specimens. No heterologous flavivirus was particularly affected by false-positive detection, suggesting that false-positive results did not result from unspecific binding of assay oligonucleotides (Table 1). Instead, false-positive results hint at the possibility of laboratory contamination potentially resulting from virus isolation attempts or PCR amplicons generated during prior Zika virus experimentation.

EQA performance varied according to the way viral RNA was prepared. The 8 laboratories conducting Zika virus detection using automated platforms performed

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Table 1. External quality assessment of 15 laboratories from 7 states of molecular diagnostic testing for Zika virus, Brazil*

<table>
<thead>
<tr>
<th>Zika Virus, copies/mL†</th>
<th>Lab ID</th>
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<th>MRS, 7.0 x 10^3</th>
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<th>DENV-2</th>
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<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4/12</td>
</tr>
<tr>
<td>15</td>
<td>+</td>
<td>(-)</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>(-)</td>
<td>(+)</td>
<td>(-)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>3/9</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>+</td>
<td>+</td>
<td>NT</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>4/12</td>
</tr>
</tbody>
</table>

Specificity was determined as positive samples containing the highest Zika virus loads were tested correctly at statistical significance ($p = 1.00$ and $p = 0.134$, respectively). Detection of all other samples showed $p$ values of <0.001. All laboratories except 1 used an assay published by Lanciotti et al. (2). CHIKV, chikungunya virus; DENV-2, dengue virus 2; DENV-4, dengue virus 4; JEV, Japanese encephalitis virus; NT, not tested; S, sample not; SLEV, St. Louis encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; +, correct positive result; –, correct negative result; †, false-positive; ‡, false-negative.

*This laboratory used the RealStar Zika Virus RT-PCR Kit (Altona Diagnostics, Hamburg, Germany).
†Positive samples contained different amounts of Zika virus strain MRS_OYP_Martinique_PaRi_2015 (representing the Asian lineage, including the outbreak strain in the Americas) or Zika virus strain MR766 (representing the African lineage). Zika virus–negative controls contained human plasma, CHIKV, DENV serotypes 2 and 4, JEV, SLEV, WNV, or YFV. Samples were prepared from 0.2 mL phosphate buffered saline supplemented with 20% human plasma and spiked with virus culture supernatants. Viruses were heat inactivated before lyophilization. Human plasma was tested negative for viral RNA and for real-time reverse transcription PCR (RT-PCR) inhibition before spiking of viral cell culture supernatants. Detection of different samples was analyzed by the exact test of goodness-of-fit with $p > 0.1$ being significant. The parameter value defining the expected ratio of correct tests was set to 0.99. Only the 2 samples containing the highest Zika virus loads were tested correctly at statistical significance ($p = 1.00$ and $p = 0.134$, respectively). Detection of all other samples showed $p$ values of <0.001. All laboratories except 1 used an assay published by Lanciotti et al. (2). CHIKV, chikungunya virus; DENV, dengue virus; ID, identification number; JEV, Japanese encephalitis virus; NT, samples not tested; S, sample not; SLEV, St. Louis encephalitis virus; WNV, West Nile virus; YFV, yellow fever virus; +, correct positive result; –, correct negative result; †, false-positive; ‡, false-negative.
generally superior (n = 8; Youden index, 0.661) compared with the 7 laboratories conducting manual RNA extraction (Youden index, 0.446) (Table 2). This finding might indicate an increased risk for contamination during manual RNA preparation. However, automated RNA preparation also might represent a proxy for more affluent settings of those laboratories.

As previously reported (3), RNA extraction critically influences the clinical lower limit of detection (LOD). Although all participants used highly sensitive real-time RT-PCRs, clinical LODs varied considerably because of different RNA extraction protocols (Figure 1, panel B). Lack of detection of low-concentration EQA samples is thus not surprising because even a small decrease in sensitivity readily causes clinical LODs above the concentration of the lowest EQA panel specimen (Figure 1, panel C). This finding highlights that optimized RNA extraction protocols are crucial for sensitive Zika virus diagnostics.

Quantification of Zika virus loads did not differ significantly between use of the armored RNA and the WHO Zika virus standard, with only 0.76 log_{10} median deviation between results (p = 0.429 by Wilcoxon signed rank test). This observation suggests usability of the armored RNA for Zika virus quantification in tropical regions. Irrespective of the standard, viral load determinations among laboratories were comparable with 0.12–0.88 log_{10} median deviations of viral load estimates among laboratories for individual Zika virus specimens. However, we also observed drastic deviations of up to 6 orders of magnitude (Figure 2), suggesting that caution must be taken upon comparing viral load determinations as markers for severe Zika virus disease (8,9) among different laboratories.

Conclusions
Some laboratories in Brazil showed suboptimal sensitivity and specificity of Zika virus diagnostic testing. However, these laboratories performed comparably to those in Europe (6). Neither sensitivity nor specificity differed significantly between laboratories in Brazil compared with those...
in Europe (p = 0.767 and p = 0.324, respectively, by Fisher exact test). Similarly, the proportion of perfectly performing laboratories in this EQA (27%) was comparable with previous EQAs of flavivirus molecular diagnostics, including yellow fever virus (18%), dengue virus (24%), and West Nile virus (27%) (10–12). Flavivirus molecular diagnostics are thus generally challenging and benefit greatly from controls, such as those provided in this EQA. This study underscores the need to combine RT-PCR and serologic testing in Zika virus diagnostic testing, despite their inherent limitations (3).

Independent of the challenges of Zika virus molecular detection, because of taxation and distributor margins, RT-PCR reagents in Latin America are usually 100%–200% more expensive than in affluent countries (13). Limited resources and relatively higher costs potentially force laboratories in Brazil to seek inferior, more affordable solutions. Enhanced access of laboratories in tropical regions to state-of-the-art reagents is thus an unresolved key component of outbreak response. Further EQAs in Brazil should involve state laboratories that carry a large proportion of Zika virus testing within the public health care system. Unfortunately, the state laboratories we contacted for this EQA could not participate because of limited resources.

Finally, lack of sensitivity directly affects estimates of the absolute risk for Zika virus–induced congenital disease upon maternal infection during pregnancy (14). False-positive results potentially have dramatic consequences for patients, as illustrated by a >90% increase in illegal abortion requests in Latin America during the 2016 Zika virus epidemic (15). Our results emphasize the need for continuous quality assessments of Zika virus diagnostic testing globally.

Table 2. Viral RNA preparation of individual laboratories in an external quality assessment for Zika virus molecular diagnostic testing, Brazil*

<table>
<thead>
<tr>
<th>Lab ID</th>
<th>Extraction method</th>
<th>Extraction kit</th>
<th>Input volume, mL</th>
<th>Elution volume, mL</th>
<th>PCR template volume, mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN, São Paulo, Brazil)</td>
<td>140</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>200</td>
<td>200</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Automated</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>200‡</td>
<td>60</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>Automated</td>
<td>Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega, São Paulo, Brazil)</td>
<td>140</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>140</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>Automated</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>200</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>140</td>
<td>60</td>
<td>4.5</td>
</tr>
<tr>
<td>8</td>
<td>Automated</td>
<td>Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega)</td>
<td>150</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>Manual</td>
<td>High Pure Viral Nucleic Acid Kit (Roche, São Paulo, Brazil)</td>
<td>200</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>160</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>Automated</td>
<td>NucliSENS easyMAG Kit (bioMérieux, Rio de Janeiro, Brazil)</td>
<td>200‡</td>
<td>50</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>Automated</td>
<td>Magna Pure Compact Nucleic Acid Isolation Kit I—Large Volume (Roche)</td>
<td>200‡</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>13</td>
<td>Automated</td>
<td>Maxwell 16 Viral Total Nucleic Acid Purification Kit (Promega)</td>
<td>100</td>
<td>50</td>
<td>5</td>
</tr>
<tr>
<td>14</td>
<td>Manual</td>
<td>QIAmp Viral RNA Mini Kit (QIAGEN)</td>
<td>140</td>
<td>60</td>
<td>5</td>
</tr>
<tr>
<td>15</td>
<td>Automated</td>
<td>Abbot mSample Preparation System RNA (4 × 24 prep) (Abbott, São Paulo, Brazil)</td>
<td>200</td>
<td>80</td>
<td>5</td>
</tr>
</tbody>
</table>

*All details are listed as declared by the participants. ID, identification.
‡Laboratory that filled the 200 µL provided in this external quality assessment panel to higher standard extraction input volumes ranging from 500 µL to 1,200 µL using human plasma tested negative for arboviral infections beforehand.
‡Laboratory that filled the 200 µL provided in this external quality assessment panel to higher standard extraction input volumes ranging from 500 µL to 1,200 µL using sterile nuclease-free water.
Acknowledgments

We thank Michaela Josten, Monika Eschbach-Bludau, and Stefan Holtin for technical support.

The Zika virus armored RNA standard is available for noncommercial use for quality assurance at the EVAg portal (https://www.european-virus-archive.com/)

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References


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Abigail L. Carlson, Daniel M. Pastula, Amy J. Lambert, J. Erin Staples, Atis Muehlenbachs, George Turabelidze, Charles S. Eby, Jesse Keller, Brian Hess, Richard S. Buller, Gregory A. Storch, Kathleen Byrnes, Louis Dehner, Nigar Kirmani, F. Matthew Kuhlmann

Heartland virus is a suspected tickborne pathogen in the United States. We describe a case of hemophagocytic lymphohistiocytosis, then death, in an immunosuppressed elderly man in Missouri, USA, who was infected with Heartland virus.

Heartland virus (HRTV; genus Phlebovirus, family Phenuiviridae [previously Bunyaviridae]) is a suspected tickborne pathogen in the United States (1). The virus was initially identified in 2009, and 9 cases of HRTV disease have been reported in the literature (2–5). Despite common features, the full spectrum of illness is unknown. We describe a fatal case of HRTV infection with hemophagocytic lymphohistiocytosis (HLH).

The Case
An elderly man from central Missouri, USA, came to the emergency department of a local hospital in June (year redacted) reporting 6 days of nausea, anorexia, and fatigue, followed by confusion and shortness of breath with cough. He denied fever, chills, or chest pain. He worked outdoors and had numerous tick exposures. His medical history included diabetes mellitus, chronic obstructive pulmonary disease, hypertension, coronary artery disease with ischemic cardiomyopathy, hypothyroidism, and rheumatoid arthritis; he was taking prednisone, methotrexate, and adalimumab.

On initial examination, he was afebrile (36.6°C), oriented only to year, and wheezed bilaterally on expiration. Laboratory results (Table 1) showed acute kidney injury, transaminitis, and mixed anion-gap metabolic acidosis and respiratory alkalosis. Initial complete blood count results showed normocytic anemia and thrombocytopenia. Total leukocyte count was within reference range, but lymphocyte count showed absolute lymphopenia. Troponin I was mildly elevated without electrocardiographic changes. Results of chest radiography and noncontrast computed tomography of the head were unremarkable. He was transferred to a tertiary care center for management of possible acute coronary syndrome and exacerbation of chronic obstructive pulmonary disease.

On post–symptom onset day (PSOD) 8, the patient became febrile (38.9°C) and increasingly confused; we intubated him for airway protection. We empirically prescribed vancomycin, meropenem, ampicillin, and acyclovir for meningoencephalitis, as well as doxycycline for possible ehrlichiosis (Figure 1). We administered a platelet transfusion to complete a lumbar puncture safely. Lumbar puncture results revealed a mildly elevated cerebrospinal fluid (CSF) protein of 56 mg/dL and an unremarkable CSF glucose level of 64 mg/dL. Specimen tubes 1 and 4 cell counts were, respectively, 14 and 0 leukocytes/µL and 158 and 14 red blood cells/µL.

Initial testing for an infectious etiology of the illness was negative (Table 2), including a low positive rickettsia IgG titer, for which repeated testing was negative. Chest radiograph on PSOD 11 showed new multifocal infiltrates; a tracheal aspirate grew Stenotrophomonas maltophilia in culture, and we started levofloxacin. On the same day, we documented leukopenia, and a core bone marrow biopsy demonstrated hypocellularity for his age without blasts, dysplasia, or atypia. We were unable to obtain an aspirate sample. We suspected HLH; his ferritin had increased from 6,308 ng/mL on PSOD 8 to 53,666 ng/mL on PSOD 11 (reference 22–322 ng/dL). In addition, he had fever, leukopenia, thrombocytopenia, and hypertriglyceridemia (Table 1), meeting at that time 4 of 5 required diagnostic criteria by the HLH-2004 Histiocyte Society guidelines (6). We initiated presumptive HLH treatment with etoposide and high-dose dexamethasone on PSOD 12. We stopped vancomycin and meropenem on PSOD 18 but restarted on PSOD 20 to
Table 1. Selected laboratory values for immunocompromised patient infected with Heartland virus, Missouri, USA*

<table>
<thead>
<tr>
<th>Test type</th>
<th>Reference range</th>
<th>4 mo before symptom onset</th>
<th>Post–symptom onset day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Leukocyte count, × 10^9 cells/µL</td>
<td>3.8–9.8</td>
<td>13.7</td>
<td>5.8</td>
</tr>
<tr>
<td>Absolute neutrophil count, cells/µL</td>
<td>1,800–6,600</td>
<td>7,400</td>
<td>5,000</td>
</tr>
<tr>
<td>Absolute lymphocyte count, cells/µL</td>
<td>1,200–3,300</td>
<td>3,300</td>
<td>700</td>
</tr>
<tr>
<td>Hemoglobin, g/dL</td>
<td>13.8–17.2</td>
<td>12.1</td>
<td>11.8</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>40.7–50.3</td>
<td>36.4</td>
<td>32.5</td>
</tr>
<tr>
<td>Platelets, × 10^3/µL</td>
<td>140–440</td>
<td>202</td>
<td>76</td>
</tr>
<tr>
<td>International normalized ratio</td>
<td>0.90–1.20</td>
<td>1.06</td>
<td>1.0</td>
</tr>
<tr>
<td>Partial thromboplastin time, s</td>
<td>25.0–37.0</td>
<td>46</td>
<td>40</td>
</tr>
<tr>
<td>Lactate dehydrogenase, units/L</td>
<td>100–250</td>
<td>NR</td>
<td>422</td>
</tr>
<tr>
<td>Haptoglobin, mg/dL</td>
<td>27–220</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Ferritin, ng/mL</td>
<td>22–322</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Fibrinogen, mg/dL</td>
<td>170–400</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>Sodium, mmol/L</td>
<td>135–145</td>
<td>139</td>
<td>128</td>
</tr>
<tr>
<td>Potassium, mmol/L</td>
<td>3.3–4.9</td>
<td>4.0</td>
<td>5.1</td>
</tr>
<tr>
<td>Carbon dioxide, mmol/L</td>
<td>22–32</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>Blood urea nitrogen, mg/dL</td>
<td>8–25</td>
<td>16</td>
<td>90</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.70–1.30</td>
<td>1.31</td>
<td>3.38</td>
</tr>
<tr>
<td>Troponin I, mg/mL</td>
<td>0.00–0.03</td>
<td>2.55</td>
<td>0.76</td>
</tr>
<tr>
<td>Cholesterol, total, mg/dL</td>
<td>0–200</td>
<td>258</td>
<td>115</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>0–150</td>
<td>426</td>
<td>532</td>
</tr>
<tr>
<td>Aspartate aminotransferase, units/L</td>
<td>11–47</td>
<td>32</td>
<td>231</td>
</tr>
<tr>
<td>Alanine aminotransferase, units/L</td>
<td>7–53</td>
<td>17</td>
<td>186</td>
</tr>
<tr>
<td>Alkaline phosphatase, units/L</td>
<td>38–126</td>
<td>85</td>
<td>60</td>
</tr>
<tr>
<td>Bilirubin, total, mg/dL</td>
<td>0.3–1.1</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>Bilirubin, direct, mg/dL</td>
<td>0.0–0.3</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Amylase, units/L</td>
<td>28–100</td>
<td>NR</td>
<td>234</td>
</tr>
<tr>
<td>Lipase, units/L</td>
<td>0–99</td>
<td>NR</td>
<td>578</td>
</tr>
<tr>
<td>pH</td>
<td>7.35–7.45</td>
<td>NR</td>
<td>7.31</td>
</tr>
<tr>
<td>PCO₂, mm Hg</td>
<td>34–45</td>
<td>NR</td>
<td>21</td>
</tr>
<tr>
<td>PₐO₂, mm Hg</td>
<td>80–105</td>
<td>NR</td>
<td>125</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>35.5–38.3</td>
<td>NR</td>
<td>36.6</td>
</tr>
<tr>
<td>F₂O₂</td>
<td>0.21</td>
<td>NR</td>
<td>0.40</td>
</tr>
</tbody>
</table>

*F₂O₂, fraction of inspired oxygen; NR, not reported; P₂CO₂, arterial partial pressure of carbon dioxide; PₐO₂, arterial partial pressure of oxygen.†On continuous venovenous hemodialysis.

Figure 1. Chronology of selected laboratory findings and therapeutic interventions for immunocompromised patient infected with Heartland virus, Missouri, USA. Gray bars indicate treatments administered. CVVHD, continuous veno-venous hemodialysis; IV, intravenous.
treat suspected sepsis after hypothermia and hypotension developed. The same day, we started voriconazole therapy to treat the patient for *Aspergillus terreus* identified from a sputum culture taken on PSOD 9. *A. terreus* had been deemed a contaminant, but we subsequently chose to treat it as a pathogen because of the patient’s leukopenia and respiratory failure. On PSOD 20, the Centers for Disease Control and Prevention (Fort Collins, CO, USA) notified the clinical care team that a blood sample obtained on PSOD 14 was positive for HRTV RNA by real-time PCR assay primers and probes as previously described (4). However, the earlier bone marrow core biopsy had extensive HRTV antigen identified by retrospectively performed IHC (Figure 2). Autopsy specimens of blood, lymph nodes, and spleen were positive for HRTV RNA by RT-PCR.

The CSF (tube 4) and blood samples obtained on PSOD 8 were analyzed retrospectively for HRTV by using reverse transcription PCR; SFG, spotted fever group.

### Conclusions

HRTV was first identified in 2009, when 2 Missouri farmers who had been bitten by ticks were admitted to a hospital for fever, fatigue, and anorexia (3). Since then, descriptions of ≥7 additional cases, including 2 deaths, have been published (2,4,5). HRTV is believed to be transmitted by

### Table 2. Infectious disease testing of immunocompromised patient infected with Heartland virus, Missouri, USA*

<table>
<thead>
<tr>
<th>PSOD</th>
<th>Test and sample type</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Aerobic culture, urine</td>
<td>Nonsignificant growth</td>
</tr>
<tr>
<td>7</td>
<td><em>Rickettsia</em> SFG IgG, serum</td>
<td>1:64 (normal &lt;1:64)</td>
</tr>
<tr>
<td>7</td>
<td><em>Rickettsia</em> SFG IgM, serum</td>
<td>&lt;1:64 (normal &lt;1:64)</td>
</tr>
<tr>
<td>7</td>
<td>HIV 1, 2 antibody, serum</td>
<td>Negative</td>
</tr>
<tr>
<td>7</td>
<td>Epstein-Barr viral capsid antibody, IgM, serum</td>
<td>Nonreactive</td>
</tr>
<tr>
<td>8</td>
<td>Aerobic and anaerobic culture, blood × 2</td>
<td>No growth</td>
</tr>
<tr>
<td>8</td>
<td><em>Ehrlichia</em> and <em>Anaplasma</em> PCR, blood</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Enterovirus RT-PCR, CSF</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Cytomegalovirus PCR, CSF</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>West Nile virus IgG, CSF</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>West Nile virus IgM, CSF</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Cryptococcal antigen, CSF</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Fungal culture, CSF</td>
<td>No growth</td>
</tr>
<tr>
<td>8</td>
<td>Aerobic culture, blood</td>
<td>No growth</td>
</tr>
<tr>
<td>9</td>
<td>Fungal culture, blood</td>
<td>No growth</td>
</tr>
<tr>
<td>9</td>
<td>Aerobic culture, tracheal aspirate</td>
<td><em>Aspergillus terreus</em></td>
</tr>
<tr>
<td>10</td>
<td>Aerobic culture, urine</td>
<td>No growth</td>
</tr>
<tr>
<td>10</td>
<td>Aerobic and anaerobic culture, blood × 2</td>
<td>No growth</td>
</tr>
<tr>
<td>10</td>
<td>Acid-fast bacilli culture, blood</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Fungal culture, blood</td>
<td>No growth</td>
</tr>
<tr>
<td>10</td>
<td><em>Ehrlichia</em> and <em>Anaplasma</em> PCR, blood</td>
<td>Not detected</td>
</tr>
<tr>
<td>10</td>
<td>Cytomegalovirus PCR, blood</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td>Histoplasma antigen, urine</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td><em>Aspergillus</em> galactomannan antigen, blood</td>
<td>Negative</td>
</tr>
<tr>
<td>10</td>
<td><em>Rickettsia</em> SFG IgG, serum</td>
<td>1:64 (normal &lt;1:64)</td>
</tr>
<tr>
<td>10</td>
<td><em>Rickettsia</em> SFG IgM, serum</td>
<td>1:64 (normal &lt;1:64)</td>
</tr>
<tr>
<td>11</td>
<td>Aerobic culture, tracheal aspirate</td>
<td>≥100,000 colonies/mL <em>Stenotrophomonas maltophilia</em>, ≥100,000 colonies/mL yeast</td>
</tr>
<tr>
<td>14</td>
<td>Heartland virus RT-PCR, blood</td>
<td>Positive</td>
</tr>
<tr>
<td>20</td>
<td>Aerobic and anaerobic culture, blood</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>20</td>
<td>Fungal culture, blood</td>
<td>Not detected</td>
</tr>
<tr>
<td>20</td>
<td>Cytomegalovirus PCR, blood</td>
<td>Negative</td>
</tr>
<tr>
<td>Autopsy</td>
<td>Heartland virus RT-PCR, blood</td>
<td>Positive</td>
</tr>
<tr>
<td>Autopsy</td>
<td>Heartland virus RT-PCR, lymph node</td>
<td>Positive</td>
</tr>
<tr>
<td>Autopsy</td>
<td>Heartland virus RT-PCR, spleen</td>
<td>Positive</td>
</tr>
</tbody>
</table>

*Positive findings are in boldface type. CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; PSOD, post–symptom onset day; RT-PCR, reverse transcription PCR; SFG, spotted fever group.
First, 4 HLH criteria were met on PSOD 8, before symptom onset day 11 shows extensive positive staining for HRTV antigen, including erythrophagocytosis by an HRTV-antigen–positive cell (arrowhead). Scale bar indicates 20μm.

Figure 2. Immunohistochemistry of bone marrow from immunocompromised patient infected with Heartland virus (HRTV), Missouri, USA. Testing of biopsied sample from post–symptom onset day 11 shows extensive positive staining for HRTV antigen, including erythrophagocytosis by an HRTV-antigen–positive cell (arrowhead). Scale bar indicates 20μm.

the lone star tick (*Amblyomma americanum*) and may be present in various mammals (7–9). This patient’s condition was similar to those described in the literature, who had fatigue, anorexia, thrombocytopenia, and transaminitis at hospital admission.

HLH is a syndrome of T-cell and macrophage hyperactivation, leading to elevated cytokines and end-organ dysfunction (10). Secondary HLH is often precipitated by infection, although malignancy and autoimmune diseases are also common precipitants. The HLH-2004 Histiocyte Society guidelines provide 8 diagnostic criteria for the syndrome, 5 of which must be met to establish the diagnosis (6). However, these guidelines were written on the basis of pediatric case series, and controversy remains regarding their sensitivity, specificity, and applicability in adults with HLH (11–13). We identified 4 HLH criteria at the time of treatment: fever, bcytopenia, hypertriglyceridemia, and hyperferritinemia. Two additional criteria, splenomegaly and hemophagocytosis, were documented at autopsy. Tests were not done for natural killer cell activity or soluble CD25 receptor levels.

We cannot directly prove that HRTV infection led to HLH in this case; however, there is a probable association. First, 4 HLH criteria were met on PSOD 8, before the identification of other infections (e.g., *S. malophilia* pneumonia and candidemia), although these conditions may have contributed to the HLH clinical course once present. Second, HRTV without *Candida* spp. was detectable in the bone marrow at the time HLH was diagnosed, and erythrophagocytosis by HRTV antigen–positive cells in bone marrow were seen in the retrospective IHC analysis (Figure 2). Finally, 1 prior HRTV case report also detected hemophagocytosis in a lymph node (4).

This patient’s severe disseminated HRTV infection may have been exacerbated by his immunosuppressant medications, co-infections, or underlying conditions and could have been further exacerbated by etoposide and dexamethasone treatment. Multiple underlying conditions were also noted in another reported patient with fatal HRTV disease (4). We detected HRTV RNA in this patient’s CSF by RT-PCR, which may reflect CNS dissemination or may be from contamination with blood during the lumbar puncture. Further investigation is necessary to determine if HRTV can invade the CNS.

Increasing recognition of HRTV disease will support generating further data on clinical characteristics of and risk factors for higher severity. Clinicians should be alert to the possibility of severe HRTV disease, including the potential development of HLH, in persons who are immunosuppressed, have multiple concurrent conditions, or both. Early recognition of HLH, treatment of patients diagnosed with this condition, and referral to tertiary care centers should be considered in these situations.

About the Author
Dr. Carlson is a physician who specializes in infectious diseases and is an associate hospital epidemiologist at the Veterans Affairs St. Louis Health Care System and Washington University School of Medicine in St. Louis, Missouri. Her research interests include antimicrobial stewardship, infection control, and health security.

References
Heartland Virus in Immunocompromised Patient


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August 2014: Vectorborne Diseases

- Leptospirosis-Associated Hospitalizations, United States, 1998–2009
- Independent Origin of Plasmodium falciparum Antifolate Super-Resistance, Uganda, Tanzania, and Ethiopia
- Global and Local Persistence of Influenza A(H5N1) Virus
- Human Exposure to Live Poultry and Psychological and Behavioral Responses to Influenza A(H7N9), China
- Rapid Whole-Genome Sequencing for Surveillance of Salmonella enterica Serovar Enteritidis
- Borrelia crocidurae Infection in Acutely Febrile Patients, Senegal
- Shelter Dogs as Sentinels for Trypanosoma cruzi Transmission across Texas, USA
- Natural Intrauterine Infection with Schmallenberg Virus in Malformed Newborn Calves
- Role of Migratory Birds in Spreading Crimean-Congo Hemorrhagic Fever, Turkey
- Isolation of MERS Coronavirus from Dromedary Camel, Qatar, 2014
- New Introductions of Enterovirus 71 Subgenogroup C4 Strains, France, 2012
- Severe Fever with Thrombocytopenia Syndrome Virus in Ticks Collected from Humans, South Korea, 2013
- Infection with Possible Precursor of Avian Influenza A(H7N9) Virus in a Child, China, 2013
- Dengue Virus Transmission by Blood Stem Cell Donor after Travel to Sri Lanka, 2012
- Severe Murine Typhus with Pulmonary System Involvement
- Detection of East/Central/South African Genotype of Chikungunya Virus in Myanmar, 2010
- Pulmonary Infection and Colonization with Nontuberculous Mycobacteria, Taiwan, 2000–2012

EMERGING INFECTIOUS DISEASES https://wwwnc.cdc.gov/eid/articles/issue/20/8/table-of-contents
A virus isolated from a sick horse from India in 2008 was confirmed by next-generation sequencing analysis to be equine encephalosis virus (EEV). EEV in India is concerning because several species of Culicoides midge, which play a major role in EEV natural maintenance and transmission, are present in this country.

Equine encephalosis is an arthropodborne, noncontagious disease of equids, characterized by fever and lassitude (1). For some affected horses, lack of appetite and some degree of edema have been reported as secondary complications (2). Equine encephalosis virus (EEV; family Reoviridae, genus Orbivirus) was first isolated from horses in South Africa in 1967 and was considered endemic to southern Africa until an outbreak was reported in Israel in 2008 (3). Reports of EEV circulation in Israel indicated that the virus is not limited to a particular region and raised concerns about the risk for its spread to other countries where competent hosts and vectors are present (3). A recent study examining EEV seroprevalence in Israel, Palestine, and Jordan has emphasized the potential risk for invasion of pathogens into new ecologic niches (4).

Two species of midge, Culicoides imicola (species complex) and C. bolitinos, have been implicated as EEV vectors (1). Although EEV infection results in high (60%–70%) morbidity rates among equids, deaths from this infection are rare (1). Diagnosis of EEV infection is confirmed by several techniques, including virus isolation in baby hamster kidney cells or suckling mice and demonstration of EEV antigen by antigen capture ELISA and serum virus neutralization tests. In addition, the TaqMan minor groove binder probe reverse transcription PCR has been used for rapid detection of EEV strains and differentiation from African horse sickness virus (5).

The EEV genome consists of 10 double-stranded RNA segments encapsulated by a double-layered icosahedral shell. The viral genome encodes 7 structural proteins (virus capsid proteins [VPs] 1–7) and 3 nonstructural proteins (NS1–3). On the basis of cross-neutralizing antibodies, 7 distinct serotypes of EEV (EEV-1–7) have been characterized (6).

Recent reports of next-generation sequencing analysis assert an unbiased identification of novel viruses in clinical samples (7,8). We investigated a virus isolated from necropsy samples received in 2008 from a horse in Pune, Maharashtra, India. Data analyzed from the MiniSeq (https://sapac.illumina.com/?langsel=/in/) output led to identification and generation of the complete EEV genome.

The Study
In July 2008, the National Institute of Virology in Pune received necropsy samples from a horse in Pune, including blood and organ tissue (lung, liver, kidney, and spleen). In addition, nasal swab, blood, and serum samples were collected from 13 horses on the same farm, reported to have clinical signs similar to those of the dead horse (fever, nasal discharge, loss of appetite, and weakness). These 13 horses recovered. No further specific details could be retrieved after this virus was identified.

All samples from the 14 horses were negative for equine influenza, Japanese encephalitis, and West Nile virus RNA according to PCR (9). We isolated virus by using horse tissue suspensions and blood samples as inocula. Each specimen was individually inoculated at a volume of 0.1 mL into 24-well plates containing a subconfluent monolayer of Vero CCL-81 cells. After incubating for 1 h at 37°C, the inoculum suspensions were removed and the cells rinsed twice with phosphate-buffered saline. We subsequently added Eagle minimum essential medium supplemented with 2% fetal calf serum and incubated the cells at 37°C. Postinfection cytopathic effects were observed by using an inverted light microscope (Nikon, Melville, NY, USA), and cellular morphologic changes were recorded. We determined viral titers in Vero CCL-81 cells to be $10^{7.67}$ 50% tissue culture infective dose/mL by using the method of Reed and Muench (10). We checked 8 vertebrate and 1 invertebrate cell lines for susceptibility to this virus. Most cells, with the exception of bat embryonic cells and A549 (human lung carcinoma), displayed postinfection cytopathic effects (Table 1). Because the initial identification attempts in 2008 yielded negative results, we performed a pathogen discovery protocol by using our recently acquired next-generation sequencing instrument. We performed DNA and RNA extractions separately on 1 mL of tissue culture supernatants or cell pellets of virus-infected cells by using a QIAamp
DNA extraction kit or QIAamp Viral RNA extraction kit (QIAGEN, Valencia, CA, USA), according to manufacturer’s instructions. Concentrations of extracted RNA were quantified by using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and host ribosomal RNA was depleted by using the NEBNext rRNA Depletion Kit (New England Biolabs, Ipswich, MA, USA). We requantified this purified RNA and prepared RNA libraries by using TruSeq Stranded mRNA Library Preparation Kit (Illumina, San Diego, CA, USA). Similarly, we prepared paired-end DNA sequencing libraries from DNA samples by using a Nextera XT DNA Library Prep Kit (Illumina). We quantified these libraries by using a KAPA Library Quantification Kit (Kapa Biosystems; Roche Diagnostics Corporation, Indianapolis, IN, USA) per manufacturer’s protocol and loaded them on an Illumina Miniseq next-generation sequencing platform. We imported the raw RNA data of 229 and 170 megabytes, along with DNA data of 176 and 199 megabytes for blood and lung samples, respectively, into the CLC Genomics Workbench software (QIAGEN) for analysis.

To assemble contiguous sequences (contigs) by using de novo assembly, we used paired-end reads for DNA and RNA. DNA reads for blood and lung samples gave 166 and 113 contigs, with average lengths of 1,112 and 6,413 bp, respectively; 29 and 1,077 contigs were generated from the assembly of RNA reads for blood and lung samples, with average lengths of 1,376 and 1,034 bp, respectively. Figure 1 depicts data from the RNA reads, classified by using Taxonomer (11). BLAST (http://blast.ncbi.nlm.nih.gov/Blas.t.cgi) analysis of RNA contigs led to sample identification as EEV, and DNA contigs failed to identify any virus.

After the infectious agent was identified, we conducted reference sequence mapping for the RNA reads by using the EEV isolate Potchefstroom with the help of a reference-guided assembly program (CLC Genomics Workbench). We recovered the full-genome sequence of EEV by using both contigs generated from de novo assembly and the reference mapping of the reads. A total of 7 fragments encoding the structural genes VP1–7 and 3 fragments encoding the genes NS1–3, together comprising ≈19,290 nt of EEV, were generated. We compared the obtained genome sequence with other publicly available EEV sequences (Table 2; Figure 2, panels A, B). NS3 gene analysis revealed that the EEV isolate from India shared the highest identity with the Potchefstroom EEV strain from South Africa, belonging to serotype 6. However, the VP2 gene had the highest resemblance to the Bryanston EEV strain, belonging to serotype 4.

Conclusions
The complete genome of the EEV isolated from a dead horse confirmed the presence of EEV in India. We compared the resultant full-genome sequence of this isolate with other EEV sequences available in GenBank. Phylogenetic analysis of the VP2 gene sequences, which is used for genotype classification of reoviruses, revealed that the isolate from India groups with the Bryanston EEV strain, belonging to serotype 4.

Table 1. Susceptibility of vertebrate and invertebrate cells to equine encephalosis virus*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Media used</th>
<th>No. cell passages</th>
<th>CPE on PID, passage 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vero CCL-81</td>
<td>MEM</td>
<td>16</td>
<td>PID 2</td>
</tr>
<tr>
<td>Bat embryo (Pipistrellus ceylonicus)</td>
<td>DMEM</td>
<td>66</td>
<td>No CPE</td>
</tr>
<tr>
<td>PS (porcine stable kidney)</td>
<td>MEM</td>
<td>107</td>
<td>PID 2</td>
</tr>
<tr>
<td>BHK-21 (baby hamster kidney)</td>
<td>MEM + 5% TPB</td>
<td>83</td>
<td>PID 2</td>
</tr>
<tr>
<td>SW-13 (human adrenal cortex)</td>
<td>L-15</td>
<td>42</td>
<td>PID 2</td>
</tr>
<tr>
<td>RD (rhabdomyosarcoma)</td>
<td>MEM</td>
<td>60</td>
<td>PID 2</td>
</tr>
<tr>
<td>C/5a (Aedes albopictus mosquito–derived)</td>
<td>MM</td>
<td>147</td>
<td>PID 7</td>
</tr>
<tr>
<td>Vero E6</td>
<td>MEM</td>
<td>53</td>
<td>PID 2</td>
</tr>
<tr>
<td>A549 (human lung carcinoma)</td>
<td>Ham F-12K</td>
<td>86</td>
<td>No CPE</td>
</tr>
</tbody>
</table>

*CPE, cytopathic effect; DMEM, Dulbecco modified Eagle medium; MEM, Eagle minimal essential medium; PID, postinfection day; TBP, tryptose phosphate broth.

Figure 1. Sample identification by use of next-generation sequencing. RNA reads from blood (A) and lung (B) samples from horse that died of equine encephalosis in India, 2008. Samples were categorized by using Taxonomer software (11); 0.5% of the reads from blood samples (total reads 2,610,292, average length 137 bp) and 12.5% of the reads from lung samples (total reads 2,125,678, average length 135 bp) were matched to those of reoviruses.
Because no specific treatments or vaccines for EEV are available, infected horses are given supportive treatment with nonsteroidal antiinflammatory drugs. The key control measure against EEV is to restrict its spread via arthropod vectors by carefully managing horses in a stable and adhering to appropriate biosafety measures. This report

| Table 2. Nucleotide and amino acid divergence of different EEV strains from the EEV isolated from a horse in India, 2008* |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| EEV GenBank sequence | NS1, 1,728 nt | NS2, 1,183 nt | NS3, 769 nt | VP1, 3,948 nt | VP2, 3,158 nt | VP3, 2,758 nt | VP4, 1,958 nt | VP5, 1,584 nt | VP6, 1,062 nt | VP7, 1,165 nt |
|                   | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD | ND  AD  ND  AD |
| EEV lungs NIV India AB811632.1 | 7 1 6 15 | 26 17 2 1 | 46 50 3 0 | 4 11 27 14 | 4 7 4 0 |
| EEV Kimron1 FJ183388.1 | 5 2 1 5 | 25 16 6 1 | 12 8 4 1 | 1 3 9 2 | 5 8 3 0 |
| EEV HS103/06 | | | | | |
| EEV lungs NIV India AB811632.1 | 7 2 4 12 | 1 2 1 0 | 47 55 7 1 | 4 10 28 17 | 4 6 7 0 |
| EEV strain 6 | 5 2 3 7 | 26 15 4 1 | 11 8 5 0 | 3 8 8 1 | 5 9 4 0 |
| EEV strain 4 | 4 2 3 10 | 25 15 5 1 | 43 47 2 0 | 4 11 26 12 | 5 7 4 0 |
| EEV strain 1 | 4 1 3 9 | 4 4 8 2 | 43 48 3 0 | 13 32 24 11 | 3 5 6 0 |
| EEV strain 2 | 4 2 7 19 | 25 15 5 1 | 46 50 5 0 | 2 6 27 15 | 5 8 2 0 |
| EEV strain 3 | 4 2 3 7 | 9 7 5 1 | 49 57 2 0 | 3 8 27 16 | 7 10 3 0 |
| EEV strain 5 | 5 2 5 13 | 9 7 6 1 | 50 59 4 0 | 2 4 28 17 | 1 2 3 0 |
| EEV strain 7 | | | | | | | | | | |
| *AD, amino acid divergence; EEV, equine encephalosis virus; ND, nucleotide divergence; NIV, National Institute of Virology, Pune, India; NS, nonstructural protein; VP, virus capsid protein. |

Figure 2. Phylogenetic tree of nonstructural 3 (A) and virus capsid protein 2 (B) genes of equine encephalosis virus. Kimura 2-parameter with (+ I) was used to create the evolutionary distance between 11 sequences of nonstructural 3 genes and virus capsid protein 2 genes from different isolates. Boldface indicates blood and lung samples from dead horse in Pune, India, 2008. GenBank accession numbers are given for reference virus sequences. Scale bars indicate nucleotide substitutions per site.
Equine Encephalosis Virus in India is concerning because several species of Culicoides midge, which play a major role in EEV natural maintenance and transmission, are present in this country. This information about EEV has been provided to the Department of Animal Husbandry, Dairying, and Fisheries of the Ministry of Agriculture and Farmers Welfare in New Delhi, India. The Department of Animal Husbandry should soon initiate a survey to provide information about EEV presence in equine centers and commercial farms and to screen samples from sick horses. The concern is that this virus may be widely circulating in India without having been noticed earlier.

Acknowledgments

We thank the authority of the horse stud farm in Pune for providing clinical samples. We also thank Rajen Lakra and Pravin Kore for their technical support during the study and Tatyana Klimova for editing this manuscript.

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References


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During October–December 2015, an epizootic hemorrhagic disease outbreak occurred in cattle in Japan. Forty-six animals displayed fever, anorexia, cessation of rumination, salivation, and dysphagia. Virologic, serologic, and pathologic investigations revealed the causative agent was epizootic hemorrhagic disease virus serotype 6. Further virus characterization is needed to determine virus pathogenicity.

Epizootic hemorrhagic disease virus (EHDV; genus Orbivirus, family Reoviridae) is an arthropodborne virus that is transmitted among ruminant hosts by the bite of Culicoides midges (1–3). EHDV infection of wild and domestic ruminants has been reported in the Americas, Africa, Asia, Australia, the Middle East, and some islands of the Indian Ocean (1) and affects primarily white-tailed deer (Odocoileus virginianus) and cattle (1–3). EHDV infection in cattle does not usually result in clinical disease, but clinical cases of epizootic hemorrhagic disease in cattle have been reported in several countries in Asia, Africa, and the Middle East (1–5).

EHDV serotype 2 (EHDV-2) strain Ibaraki virus was first identified in affected cattle in Japan in 1959. Ibaraki virus has caused disease repetitively in cattle, with signs and symptoms including fever, anorexia, nasal and ocular discharge, congestion of conjunctival and nasal mucous membranes, swollen eyelids and tongue, and dysphagia (4,6). A large-scale epidemic of atypical Ibaraki disease also occurred in Japan in 1997; the epidemic caused mainly abortion and stillbirth in cattle, and the causative agent was found to be EHDV-7 (7,8). Several strains of EHDV-1, EHDV-7, and EHDV-10 were also observed in Japan but did not appear to be associated with clinical disease in cattle (8).

In October 2015, an outbreak of febrile illness occurred in cattle in Hyogo Prefecture, Japan, and lasted 3 months. We summarize the features of this outbreak, which affected 46 cattle at 38 farms.

**The Study**

Of the 46 affected cattle, 40 were beef cattle (Japanese Black) and 6 were dairy cattle (Holstein heifers). The average age of all affected animals was 114.7 (range 11–187) months. The clinical signs observed in beef cattle were fever, anorexia, cessation of rumination, swollen eyelids, salivation, paralysis of the tongue, difficulty swallowing, nasal and ocular discharge, and abortion (Table 1). Seven beef cattle died, and 2 were euthanized because they did not recover despite treatment of symptoms. The dairy cattle showed fever, anorexia, coughing, conjunctivitis, cessation of rumination, salivation, difficulty swallowing, and reduced milk production.

Early in the outbreak, Ibaraki disease was suspected, so blood samples were collected. We washed the blood cells and used them for virus isolation and reverse transcription PCR (RT-PCR) for group-specific and serotype-specific EHDV detection (9,10). Paired serum samples were also collected from 20 of the 46 affected animals at early onset of the outbreak and after the outbreak at 1- or 2-month intervals. We used the paired serum samples for neutralization tests and conducted necropsies on 2 euthanized animals.

All 46 of the affected animals tested positive for EHDV by group-specific RT-PCR (9) and EHDV-6 by serotype-specific RT-PCR (10), although we did not isolate infectious virus. The neutralizing antibody titer of serum samples acquired after the outbreak (n = 20) were 1:4-fold in EHDV-6 strain AUS1981/07 CSIRO 753 (Table 2). The neutralization test also showed increases (≥4-fold) in EHDV-6 antibody titers for paired serum samples of 5 cattle (nos. 6, 7, 10, 11, and 13), although such increases were not observed in the other 15 cattle (Table 2).

At necropsy, we observed edema of the esophagus, pharynx, and tongue in an affected cow with dysphagia. The lumen of the esophagus was also dilated (Figure 1, panel A). The histopathologic lesions included hyaline degeneration and necrosis of striated muscle accompanied by cell infiltration in the esophagus and tongue (Figure 1, panel B), thrombosis of small vessels, a proliferation of connective tissue in the esophagus, and necrosis of striated...
muscle in the pharynx. We conducted immunohistochemical assays with necropsy tissue samples from this cow using rabbit antisera against EHDV-6 AUS1981/07 CSIRO 753 and detected antigen in the vascular endothelium of the esophageal muscle layer (Figure 1, panel C).

We further characterized the causative agent by performing sequence analysis of genome segment 2, which correlates with serotype, and segment 3, which correlates with geographic genetic type, of outbreak isolate HG-1/E/15. We designed RT-PCR primers to amplify segments 2 and 3 from the full-length cDNA on the basis of sequence data of EHDV strains from Japan and Australia available in GenBank. We performed RT-PCR using RNA obtained from an affected cow (no. 9; Table 2) as template; we then purified the RT-PCR products and subjected them to direct sequencing. We used the sequence data to analyze the phylogenetic relationships between EHDV HG-1/E/15 and other EHDV isolates.

We aligned the sequences using ClustalW (11) and constructed phylogenetic trees with MEGA5 using the neighbor-joining method (12); the reliability of the branching orders was evaluated by the bootstrap test (1,000 replicates). Sequence identities between EHDV HG-1/E/15 and the other EHDV isolates were calculated with GENETYX version 10 (GENETYX Corporation, Tokyo, Japan). The results of the phylogenetic analysis of segment 2 (DDBJ accession no. LC320035) revealed that HG-1/E/15 clustered with EHDV-6 (Figure 2, panel A) and showed highest identity to EHDV-6 AUS1981/07 CSIRO 753 (89.68% nucleotide and 93.51% amino acid identities). In contrast, the phylogenetic analysis of segment 3 (DDBJ accession no. LC320036) showed that EHDV-6 HG-1/E/15 sorted into the E1 subgroup of the Eastern group (Figure 2, panel B) (8) and had highest identity to the Ibaraki isolate EHDV-2 JPN1959/01 (95.96% nucleotide and 99.77% amino acid identities).

**Conclusions**

We determined this febrile illness affecting 46 cattle in Japan in 2015 was an epizootic hemorrhagic disease outbreak caused by EHDV-6 infection on the basis of clinical findings, RT-PCR results, neutralization tests, and sequence analyses. Clinical EHDV-6 cases in cattle have been reported in Turkey, Bahrain, Israel, Morocco, Tunisia, Algeria, and Réunion Island (3,5,13,14), but the EHDV-6...
detected in this outbreak (HG-1/E/15) clustered separately from the EHDV-6 isolates from Africa and the Middle East in the phylogenetic tree analysis of segment 2 (Figure 2, panel A). Because HG-1/E/15 clustered with EHDV isolates from Japan and Australia in the E1 subgroup in the phylogenetic analysis of segment 3 (Figure 2, panel B) (8), HG-1/E/15 appears to be derived from EHDVs circulating in the Asia-Pacific region.

The neutralization tests showed significant increases of antibody titers in 5 cattle, suggesting recent EHDV-6 infection. At the same time, no significant increase of antibody titers was observed with the other 15 cattle. These results suggest that the onset of the epizootic hemorrhagic disease outbreak might have occurred >3–5 weeks after the EHDV-6 infection in the 15 seronegative cattle, considering that neutralizing antibody titer increases have been observed to occur in cattle 11–14 to 21–37 days after experimental EHDV-6 infection (15).

The clinical and pathologic findings of this outbreak are similar to those observed for Ibaraki disease in cattle. Thus, EHDV-6 HG-1/E/15 possibly has pathogenic characteristics similar to those of Ibaraki strain EHDV-2 JPN1959/01 in cattle. Further investigations are needed to clarify the genetic characteristics of EHDV-6 HG-1/E/15 to determine why this outbreak occurred.

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**References**


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**July 2013: Vectorborne Infections**

- Transmission of *Streptococcus equi* Subspecies *zooepidemicus* Infection from Horses to Humans
- Travel-associated Illness Trends and Clusters, 2000–2010
- Quantifying Effect of Geographic Location on Epidemiology of *Plasmodium vivax* Malaria
- Babesia microti Infection, Eastern Pennsylvania, USA
- Reemergence of Chikungunya Virus in Bo, Sierra Leone
- Novel Bartonella Agent as Cause of Verruga Peruana
- Schmallenberg Virus among Female Lambs, Belgium, 2012
- Psychrobacter arenosus Bacteremia after Blood Transfusion, France
- Ciprofloxacin-Resistant *Campylobacter* spp. in Retail Chicken, Western Canada
- Asynchronous Onset of Clinical Disease in BSE-Infected Macaques
- Prevalence of Nontuberculous Mycobacteria in Cystic Fibrosis Clinics, United Kingdom, 2009
- Reducing Visceral Leishmaniasis by Insecticide Impregnation of Bed-Nets, Bangladesh
- Genetic Variants of *Orientia tsutsugamushi* in Domestic Rodents, Northern China
- MDR TB Transmission, Singapore

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https://wwwnc.cdc.gov/eid/articles/issue/19/7/table-of-contents
Fatal Visceral Leishmaniasis Caused by *Leishmania infantum*, Lebanon

Rana El Hajj, Hiba El Hajj, Ibrahim Khalifeh

Visceral leishmaniasis, a fatal disease if not treated, is caused by *Leishmania* parasites. This disease might be overlooked in the Middle East because of limited awareness and low incidence. We report 5 patients who died of visceral leishmaniasis in Lebanon and make recommendations to improve faster diagnosis and treatment.

Leishmaniasis is a parasitic disease characterized by different clinical manifestations depending on patient immune response and causative species (1). Visceral leishmaniasis, the most severe form, is fatal if untreated (2). This disease is caused by *Leishmania donovani*, which is endemic to Africa and Asia, causes anthropopotic visceral leishmaniasis, and is associated with high mortality rates (3). However, *L. infantum*, which undergoes zoonotic transmission, is associated with fewer deaths and is endemic to Latin America and the Middle East (4). This species shows a mortality rate of 6% for children (5).

In the Middle East, cutaneous leishmaniasis is the most common endemic form of leishmaniasis and is caused mainly by *L. tropica* and *L. major* (6). *L. infantum* is reported to cause cutaneous leishmaniasis and visceral leishmaniasis in Syria, but only cutaneous leishmaniasis in Lebanon (7,8). During 1958–2014, visceral leishmaniasis showed a low incidence in Syria; 17 cases were reported in 2008 and 36 cases in 2014 (9,10). However, no molecular or biochemical typing was performed to identify the causative species and strains (11). Moreover, visceral leishmaniasis caused by *L. infantum* has not been reported in Lebanon (12).

Recently, displacement of refugees during the ongoing crisis in Syria resulted in a massive population migration and spread of communicable diseases, including cutaneous leishmaniasis (6,11). In Lebanon, 2,420 families from Syria were given a diagnosis of cutaneous leishmaniasis. As of April 2017, a total of 2,057 (85%) of these families were infected with *L. tropica* and 363 (15%) with *L. major* (6). These infections indicate the need for early diagnosis of visceral leishmaniasis and prevention of deaths in the Middle East. We report visceral leishmaniasis in refugees from Syria in Lebanon who acquired *L. infantum* in Syria.

**The Study**

Five refugee children from Syria (age range 2–11 years) died of visceral leishmaniasis during 2014–2017 because of a late diagnosis and lack of awareness of this disease in Lebanon. All 5 children had migrated from the north ern coast of Syria to Lebanon and had resided in Beirut for an average of 9 months (range 7–11 months). Visceral leishmaniasis developed 4–6 months after they left Syria. Further investigations showed that the siblings of 3 of these patients were infected while staying in Syria.

All patients visited medical institutions in Lebanon and had fever, abdominal distension, ascites, hepatosplenomegaly, and pancytopenia (Table). Three patients were given incorrect diagnoses of leukemia and were given steroids and blood transfusions. The remaining 2 patients were given incorrect diagnoses of a hemophagocytic syndrome with an idiopathic etiology and treated accordingly.

These 5 patients were then reevaluated at the American University of Beirut Medical Center (Beirut, Lebanon) after an average of 4.8 months. Microscopic examination of bone marrow aspirates and smear specimens showed a few scattered amastigotes within macrophages for 2 patients (Figure).

A diagnosis of visceral leishmaniasis caused by *L. infantum* was confirmed by PCR amplification of the internal transcribed spacer 1 region of the parasite (13), followed by restriction fragment length polymorphism analysis of the internal transcribed spacer 1 region amplicon (14). This analysis specifically distinguishes *L. infantum* from other *Leishmania* species. After confirmation of visceral leishmaniasis, the patients were treated with Abelcet (amphotericin B lipid complex) (Teva Pharma BV, Harlow, UK) according to the manufacturer’s guidelines. However, the delay in diagnosis led to an advance disease stage and lack of response to treatment, followed by death.

**Conclusions**

We report 5 children among refugees from Syria in Lebanon who died of visceral leishmaniasis caused by *L. infantum*. Our report provides insightful baseline information about knowledge, practices, and control regarding this disease. The combination of hepatosplenomegaly, fever, and pancytopenia should raise the suspicion for visceral leishmaniasis and result in prompt intervention. These interventions might prevent misdiagnosis and enable appropriate treatment strategies at early stages of the disease to reduce the number of deaths.
Fatal Visceral Leishmaniasis Caused by L. infantum

About the Author
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References

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Table. Clinical characteristics of 5 patients with visceral leishmaniasis, Lebanon, 2014–2017*

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age, y/sex</th>
<th>Abdominal distension</th>
<th>Hepatosplenomegaly</th>
<th>Fever</th>
<th>Ascites</th>
<th>Blood</th>
<th>Lung infiltrates</th>
<th>Insect bite</th>
<th>Delay in diagnosis, mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11/F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pancytopenia</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>7/M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pancytopenia</td>
<td>+</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>5/M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pancytopenia</td>
<td>+</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>4/M</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pancytopenia</td>
<td>+</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>2/F</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Pancytopenia</td>
<td>+</td>
<td>–</td>
<td>4</td>
</tr>
</tbody>
</table>

*–, negative; +, positive.

Figure. Bone marrow smear sample (A) and bone marrow aspirate (B) for patient 2 with visceral leishmaniasis caused by Leishmania infantum, Lebanon. Arrows show amastigotes within macrophages. Panel A, Wright Giemsa stain, original magnification x400; panel B, hematoxylin and eosin stain, original magnification x200.
Second Human Pegivirus in Hepatitis C Virus–Infected and Hepatitis C Virus/HIV-1–Co-infected Persons Who Inject Drugs, China

Haiying Wang,1 Zhengwei Wan,1 Qiang Sun,1 Nalin Zhu, Tianyi Li, Xiuqi Ren, Xiaoping An, Shuyun Deng, Yue Wu, Xiufen Li, Lin Li, Jingyun Li, Yigang Tong, Shixing Tang

We report the presence of the second human pegivirus (HPgV-2) in Guangdong and Sichuan Provinces in China. The prevalence of HPgV-2 in hepatitis C virus/HIV-1–co-infected persons who inject drugs was 12.9% in Guangdong and 15.9% in Sichuan. This population is at high risk for HPgV-2 infection.

In 2015, the second human pegivirus (HPgV-2) was independently reported by 2 groups in the United States (1,2). Previous reports have indicated that HPgV-2 (also known as HHpgV-1) is a transfusion-transmitted virus and is associated with hepatitis C virus (HCV) infection (1–5). The distribution and prevalence of HPgV-2 infection worldwide are of great importance but remain to be determined. In this study, we demonstrate the existence of HPgV-2 in the southern province of Guangdong and southwestern province of Sichuan in China. We have also identified HCV-infected persons, in particular HCV/HIV-1 co-infected persons who inject drugs (PWID), as populations at high risk for HPgV-2 infection. In addition, our work reveals the difference in the prevalence, distribution, and phylogeny between the first human pegivirus (HPgV; formerly GB virus C or hepatitis G virus) (6,7) and HPgV-2.

The Study

In our initial investigation of HPgV-2, we screened a total of 367 delinked serum or plasma samples from high-risk groups for infection with HCV and HIV-1 and 500 healthy volunteer blood donors from Guangdong Province, China, by using ELISA (2,5), and a nested reverse transcription PCR targeting both the 5′ untranslated region and nonstructural protein 3 regions of HPgV-2 (3,5). We observed a low frequency (0.4%) of HPgV-2 antibody detection and the absence of HPgV-2 viremia in healthy blood donors tested in our study. Out of 86 HCV-infected patients, 1 (1.2%) was positive for both HPgV-2 antibodies and viral RNA (Table 1). Furthermore, we did not detect HPgV-2 RNA in men who have sex with men (MSM), although 1 (0.5%) of the 211 MSM was weakly positive for HPgV-2 antibodies and negative for HPgV-2 RNA (Table 1; Figure 1).

We observed a relatively high prevalence of HPgV-2 infection in HCV/HIV-1 co-infected PWID in Guangdong Province; 12.9% (9/70) were positive for HPgV-2 antibodies and 5.7% (4/70) for HPgV-2 RNA (Table 1). We obtained similar results from 270 PWID from Sichuan Province; 15.9% (43/270) were positive for HPgV-2 antibodies and 3.0% (8/270) for HPgV-2 RNA (Table 1). Using the Fisher exact test, we observed a statistically significant difference between HCV-positive and HCV-negative patients in the prevalence of having HPgV-2 antibodies (6.2% vs. 0; p<0.001) and prevalence of having HPgV-2 RNA (5% vs. 0; p = 0.026). Similarly, we observed a statistically significant difference between HIV-1–positive/HCV-positive patients and HIV-1–positive/HCV-negative patients in the prevalence of having HPgV-2 antibodies (10% vs. 0; p<0.001) and prevalence of having HPgV-2 RNA (4% vs. 0; p = 0.040) (Table 2). These findings indicate a close association between HPgV-2 and HCV infection and synergy between HIV-1 and HCV infection with respect to HPgV-2 infections (5).

Furthermore, we obtained 6 near full-length genome sequences of HPgV-2 by using next-generation sequencing or sequencing of PCR products (5). These strains from China, which included 2 from PWID (IDU31 and SC-LS-01), 2 from HCV-infected patients (HCV-121 and C346), and 2 from HCV-infected blood donors (HCV1241 and HCV1563), exhibited an identity of 93.6%–97.8% at the whole-genome level. Compared with other HPgV-2 strains from the United States and United Kingdom, the nucleotide sequence identity was 93.7%–96.2%. Sequence divergence was greatest at synonymous sites, with ratios of nonsynonymous to synonymous nucleotide substitutions of 0.125–0.150, which are consistent with

1These authors contributed equally to this article.
Second Human Pegivirus, China

Phylogenetic analysis indicated that HPgV-2 strains from China, the United States, and the United Kingdom clustered together to form a separate branch and fell into group 1 with the closely related pegiviruses from bats and rodents (Figure 2). Other pegiviruses from human, simian, and equine sources formed group 2, in which the variants of HPgV fell into a separate clade. These results illustrate the difference between the 2 human pegiviruses (1,2,8) and the low level of genetic diversity of HPgV-2 strains (1–3).

In contrast to our findings on HPgV-2 infection, we observed a high frequency of HPgV infection across all 3 populations tested (HCV-infected patients, PWID, and MSM) (Tables 1, 2). The percentage of HPgV viremia was 14.0% (14/86) in HCV-infected patients, 19.0% (40/211) in MSM, and 40.0% (28/70) in PWID (Table 1). Among MSM, the prevalence of HPgV RNA was 28.0% (28/100) in those who were infected with HIV-1 alone and 33.3% (4/12) in those who were HIV-1/HCV co-infected (Table 1). For MSM who were negative for both HIV-1 and HCV, 7.9% (7/89) were positive for HPgV RNA (Table 1).

Conclusions

We report the detection of the second human pegivirus, HPgV-2, in HCV-infected (in particular HCV/HIV-1 co-infected) persons in Guangdong and Sichuan Provinces, China (Table 1). Our results and those from previous studies demonstrate that the virus occurs in several geographically distinct regions in the world (1–4,9,10).

HPgV and HPgV-2 are the only known human pegiviruses (8), and comparing their association with HCV and HIV-1 infection is of great interest. Consistent with previous reports, we found that the prevalence of HPgV viremia was 7.9% in HCV and HIV-1–negative MSM and 33.3%–40% in HCV/HIV-1 co-infected MSM and PWID (Table 1). In contrast, only 0.5% of MSM and 0.4% of healthy blood donors were positive for HPgV-2 antibodies, but all were negative for HPgV-2 RNA (Table 1). These results indicate that HPgV-2 infection might be much less frequent than HPgV infection, possibly because of its low transmissibility or high clearance rate (2–4). The dramatic difference of distribution and prevalence between HPgV and HPgV-2 infections in different populations provides a clue for investigation of disease association with HPgV-2. HPgV does not cause human diseases (11) and can inhibit HIV-1 replication as well as prolong survival of

Table 1. Detection frequencies of HPgV-2 in different populations in Guangdong and Sichuan Provinces, China*

<table>
<thead>
<tr>
<th>Province, group, and subgroup</th>
<th>No. tested</th>
<th>Ab+, no. (%)</th>
<th>RNA+, no. (%)</th>
<th>HPgV RNA+, no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Guangdong Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCV-infected patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ab+/RNA+</td>
<td>57</td>
<td>1 (1.8)</td>
<td>1 (1.8)</td>
<td>8 (14.0)</td>
</tr>
<tr>
<td>Ab–/RNA+</td>
<td>7</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Ab+/-RNA–</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>4 (18.2)</td>
</tr>
<tr>
<td>Total</td>
<td>86</td>
<td>1 (1.2)</td>
<td>1 (1.2)</td>
<td>12 (14.0)</td>
</tr>
<tr>
<td>PWID</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1+/HCV Ab+/RNA+</td>
<td>70</td>
<td>9 (12.9)</td>
<td>4 (5.7)</td>
<td>28 (40.0)</td>
</tr>
<tr>
<td>MSM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1+/HCV+</td>
<td>12</td>
<td>1 (8.3)</td>
<td>0</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>HIV-1+/HCV–</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>28 (28.0)</td>
</tr>
<tr>
<td>HIV-1–/HCV+</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>1 (10.0)</td>
</tr>
<tr>
<td>HIV-1–/HCV–</td>
<td>89</td>
<td>0</td>
<td>0</td>
<td>7 (7.9)</td>
</tr>
<tr>
<td>Total</td>
<td>211</td>
<td>1 (0.5)</td>
<td>0</td>
<td>40 (19.0)</td>
</tr>
<tr>
<td>Blood donors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1–/HCV–/HBV–</td>
<td>500</td>
<td>2 (0.4)</td>
<td>0</td>
<td>NT</td>
</tr>
<tr>
<td>HPgV Ab+/HCV RNA+</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>NT</td>
</tr>
<tr>
<td><strong>Sichuan Province</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PWID</td>
<td>270</td>
<td>43 (15.9)</td>
<td>8 (3.0)</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Ab, antibodies; HBV, hepatitis B virus; HCV, hepatitis C virus; HPgV, human pegivirus; HPgV-2, second human pegivirus; MSM, men who have sex with men; NT, not tested; PWID, people who inject drugs; +, positive; –, negative.

Figure 1. Detection of second human pegivirus (HPgV-2) antibodies in different samples in Guangdong and Sichuan Provinces, China. Serum or plasma samples from 86 HCV-infected patients, 70 PWID, 122 MSM, and 102 blood donors (100 samples that were negative for HPgV-2 antibodies plus 2 positive samples) are included. The antibody titers from each sample are plotted on the y-axis. HPgV-2 RNA–positive samples are shown in red. HCV, hepatitis C virus; MSM, men who have sex with men; OD_{450}, optical density at 450 nm; PWID, persons who inject drugs.
HIV-1–infected and Ebola virus–infected patients (12–14). However, possible pathogenicity and disease association of HPgV-2 remain to be elucidated.

The high-risk populations susceptible to HPgV-2 infection includes HCV-infected patients and, in particular, HCV/HIV-1 co-infected PWID. Most (93.3%) of HPgV-2 infected patients were also co-infected with HCV (1–4). Notably, the relatively high frequency of HPgV-2 RNA detection was observed in HCV/HIV-1 co-infected PWID in Guangdong (5.7%) and Sichuan (3.0%) Provinces of China (Table 1) and in the United States (10.9%) (9). In contrast, a somewhat lower percentage (1.7%) of HCV-positive PWID in the United Kingdom were reported to be HPgV-2 RNA positive, whereas none of the 30 HIV-1 singly infected and 36 HCV/HIV-1 co-infected PWID were positive for HPgV-2 RNA (3). These discordant results warrant more studies in different countries to address the association between HPgV-2 and HCV/HIV-1 co-infection.

Our findings are subject to 2 limitations. First, because a limited number of samples from only 2 provinces of China were tested, the results might not represent overall prevalence of HPgV-2 infection throughout all of China. Second, this study was a cross-sectional rather than a longitudinal study, therefore, the proportions of persistent infection and natural history of HPgV-2 infection remain to be determined.

Future studies should address several questions: whether the close association between HPgV-2 and HCV infection represents a biologic dependence of these 2
viruses; how HCV/HIV-1 co-infection facilitates HPgV-2 infection; and whether HCV or HIV-1 viral proteins enhance the transmissibility or infectivity of HPgV-2. In addition, because the rarity of HPgV-2 detection in MSM could be a result of the low frequency of HCV or HIV-1 infection or the transmission route of HPgV-2, further research should aim to determine if HPgV-2 is more like a transfusion-transmitted virus rather than a sexually transmitted virus.

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Dr. Wang is a postdoctoral fellow at the School of Public Health of Southern Medical University in Guangzhou. Her research interests include identification and diagnosis of viral pathogens and investigation of viral pathogenesis.

References

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Characterization of Clinical Isolates of Bartonella henselae Strains, South Korea

Hea Yoon Kwon,1 Young Kyoungh Park,1 Sun Myoung Lee, Ji Hyeon Baek, Jae-Seung Kang, Moon-Hyun Chung, Eun Ji Kim, Jin-Soo Lee

Bartonella henselae, a gram-negative bacterium, is a common causative agent of zoonotic infections. We report 5 culture-proven cases of B. henselae infection in South Korea. By alignment of the 16S rRNA sequences and multilocus sequencing typing analysis, we identified all isolates as B. henselae Houston-1 strain, which belongs to sequence type 1.

The genus Bartonella includes infectious, gram-negative, facultative intracellular bacteria of numerous species. Among the Bartonella species, B. henselae is known as one of the most noteworthy pathogens (1). B. henselae causes cat-scratch disease, which is a common zoonosis and manifests various clinical symptoms (2).

A case of B. henselae infection in South Korea was confirmed in 2005 by PCR (3). Although a few more studies have been published after this case of B. henselae, only 2 cases were culture-proven: 1 from blood and 1 from bone marrow (4,5). Because of difficulties in cultivation and isolation, studies of the isolation of B. henselae from clinical specimens remain scarce. In this study, we analyzed the characteristics of the isolated B. henselae strains in South Korea and compared the clinical features of the patients.

The Study

We conducted the study among patients who visited Inha University Hospital, a tertiary hospital in Incheon, South Korea, during 2009–2016. From these patients, we isolated 5 cases in which B. henselae was identified from cultures of blood or bone marrow (Table 1).

Case-patient 1 (IIBC1301) was a 22-year-old man hospitalized for fever and myalgia, symptoms that had lasted for 1 month. The patient had an erythematos papular rash on her face and extremities and tenderness in her abdomen. Computed tomography (CT) of the abdomen showed chronic cholecystitis; therefore, levofloxacin and metronidazole were prescribed (online Technical Appendix Figure, panel A, https://wwwnc.cdc.gov/EID/article/24/5/17-1497-Techapp1.pdf). B. henselae was identified from cultures of blood obtained on the first day of the hospitalization. The patient had not raised any animals. After discharge, the patient experienced continuous fever, poor oral intake, and weight loss. Reevaluation showed centriflobular ground-glass opacity in both lung fields on chest CT and growth of Mycobacterium tuberculosis on sputum acid-fast bacilli culture (online Technical Appendix Figure, panel B). A pulmonary tuberculosis infection was diagnosed and treated with antituberculosis medication.

Case-patient 2 (IIBC1302) was a 40-year-old woman hospitalized for fever and myalgia, symptoms that had lasted for 1 month. The patient had an erythematos papular rash on her face and extremities and tenderness in her abdomen. Computed tomography (CT) of the abdomen showed chronic cholecystitis; therefore, levofloxacin and metronidazole were prescribed (online Technical Appendix Figure, panel A, https://wwwnc.cdc.gov/EID/article/24/5/17-1497-Techapp1.pdf). B. henselae was identified from cultures of blood obtained on the first day of the hospitalization. The patient had not raised any animals. After discharge, the patient experienced continuous fever, poor oral intake, and weight loss. Reevaluation showed centriflobular ground-glass opacity in both lung fields on chest CT and growth of Mycobacterium tuberculosis on sputum acid-fast bacilli culture (online Technical Appendix Figure, panel B). A pulmonary tuberculosis infection was diagnosed and treated with antituberculosis medication.

Case-patient 3 (IIBC1303) was a 52-year-old woman hospitalized for fever and left flank pain; her symptoms had persisted for 1 month. She also reported right-side neck swelling and pain at neck levels II, III, and VA. B. henselae was isolated from cultures of blood collected on the 16th day of hospitalization. She had no contact with animals.

Case-patient 4 (IIBC1304) was a 42-year-old man we previously reported (5) whose main complaints were fever, rash, and arthralgia. B. henselae was isolated from a bone marrow sample. The patient had no contact with or experience in raising pets.

Case-patient 5 (IIBC1305), also previously published (4), was a 73-year-old woman who had B. henselae isolated from her blood. She also did not have any contact with animals.

Bartonella species can be grown by blood agar–based culture systems. However, it is difficult to culture them in this way because the growth of bacterial cells is slow, and obtaining colonies on the agar plate takes a long time. On the other hand, Bartonella species grow more rapidly with cell culture–based systems (6). For testing of these patients, we grew ECV304 cells in M199 media containing 10% heat-inactivated fetal bovine serum and inoculated 1 mL of whole blood or other samples from the patients onto the cells. After 24 hours, we washed the cells with Dulbecco’s phosphate-buffered saline and maintained them in M199 media. We performed an immunofluorescence assay...
Isolates of *Bartonella henselae*, South Korea

Table 1. Demographic and clinical characteristics of 5 case-patients whose serum sample cultures revealed the presence of *Bartonella henselae*, South Korea*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Case-patient 1</th>
<th>Case-patient 2</th>
<th>Case-patient 3</th>
<th>Case-patient 4 (5)</th>
<th>Case-patient 5 (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y/sex</td>
<td>22/M</td>
<td>40/F</td>
<td>52/F</td>
<td>42/M</td>
<td>73/F</td>
</tr>
<tr>
<td>Clinical symptoms</td>
<td>Inguinal LAP, rash</td>
<td>Fever, myalgia</td>
<td>Febrile sense, left flank pain</td>
<td>Rash, fever, myalgia</td>
<td>Fever, general weakness</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>External iliac chain, inguinal area, supraclavicular area</td>
<td>Left neck level IV, V</td>
<td>Right neck II, III, VA</td>
<td>Right supraclavicular area</td>
<td>None</td>
</tr>
<tr>
<td>Leukocytes, cells/µL</td>
<td>10,820</td>
<td>6,130</td>
<td>8,180</td>
<td>19,260</td>
<td>5,120</td>
</tr>
<tr>
<td>AST/ALT, IU/dL</td>
<td>132/270</td>
<td>107/51</td>
<td>30/16</td>
<td>212/246</td>
<td>47/56</td>
</tr>
<tr>
<td>ESR, mm/h/CRP, mg/dL</td>
<td>21/3.93</td>
<td>44/5.5</td>
<td>4/0.14</td>
<td>25/12.9</td>
<td>22/13.16</td>
</tr>
<tr>
<td>Treatment</td>
<td>Third-generation cephalosporin, doxycycline</td>
<td>Levofloxacin, metronidazole, third-generation cephalosporin and doxycycline</td>
<td>Third-generation cephalosporin, minocycline, metronidazole</td>
<td>Doxycycline, changed to minocycline</td>
<td>Third-generation cephalosporin, doxycycline</td>
</tr>
<tr>
<td>Pets</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Co-occurring conditions</td>
<td>None</td>
<td>Pulmonary tuberculosis</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate.

(IFA) with the patient’s own serum (1:40 diluted) every week after the inoculation. When the growth of bacteria was observed, we scraped all cultured cells from the T25 flask. We then reinoculated 1 mL of infected ECV304 cells onto uninfected ECV304 cells in a T75 flask for expansion of bacterial cells.

**Figure.** Phylogenetic tree of 5 *Bartonella henselae* clinical isolates from patients in South Korea (black dots) and closely related species based on 16S rRNA gene sequences. Database accession numbers are provided for reference sequences. Scale bar indicates nucleotide substitutions per site.
To identify the bacterial isolates, we amplified and sequenced the 16S rRNA gene (7). The pathogens cultured from the specimens showed the highest sequence similarities with *B. henselae* Houston-1 strain (GenBank accession nos. KY773227, KY773228, KY773229, KY773290, and KY885188). The similarity was >99% (Figure) (8,9). IFA results using a commercial *Bartonella* IFA IgG kit (FOCUS Diagnostics, DiaSorin Molecular, Cypress, CA, USA) also showed positive results for all patients’ serum samples; titers ranged from 1:40 to 1:1,280 (Table 1). We also performed multilocus sequence typing to determine the genotypes of *B. henselae* isolates (10) and found that all isolates belonged to sequence type 1 (Table 2).

**Conclusions**

We cultured *B. henselae* isolates from clinical samples and compared characteristics of 5 patients: 3 new cases and 2 previously reported cases from which *B. henselae* was isolated (Table 2). Because of the diverse manifestations of *B. henselae* infection, the symptoms were similar to those of other bacterial infections. *B. henselae* infections in 3 patients were initially misdiagnosed as other diseases: sexually transmitted disease (case-patient 1), enteric fever-like syndrome (case-patient 2), and acute pycnophlebitis (case-patient 3). The diagnosis of *B. henselae* infection was made even more difficult because none of these 5 patients reported a history of raising cats. However, the absence of contact with animals should not preclude infection; even though *B. henselae* infection is usually related to cat scratches or bites, it may also occur without animal contacts (5). It is also noteworthy that the patient described in case 2 was co-infected with pulmonary tuberculosis. Co-infection with *B. henselae* and *Mycoplasma* spp. has also been reported in previous studies (11,12). Co-infection with other bacteria suggests that infection with *Bartonella* species may weaken the host’s immune system, leaving the host vulnerable to secondary infections. In addition, these co-infections may cause difficulty in diagnosing *Bartonella* infection.

Multilocus sequence typing indicated that all isolates from this study belonged to *B. henselae* sequence type 1. This result is consistent with previous studies, which showed relatively less diversity among human strains than among the feline reservoir (10,13).

In summary, the clinical features of *B. henselae* infection are diverse and nonspecific, which could initially lead to misdiagnosis as other diseases. Physicians and patients should consider that *Bartonella* infection presents various clinical symptoms and might be a common cause of fever of unknown origin, irrespective of exposure to cats. Once *Bartonella* infection is suspected, cell culture should be considered to confirm the diagnosis.

This work was supported by a research grant from Inha University Hospital, Incheon, Korea.

**About the Author**

Dr. Kwon is a medical doctor at Inha University Hospital in Incheon, South Korea. Her research interests include infectious diseases, especially focusing on intracellular bacteria and epidemiology. Dr. Park is a research fellow at Inha University in Incheon. Her primary research interests include antimicrobial agents and vaccines for treatment of infectious diseases.

**References**


10. Iredell J, Blanckenberg D, Arvand M, Grauling S, Feil EJ, Birtles RJ. Characterization of the natural population of

<table>
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<td>IIBC1305</td>
<td>Blood</td>
<td>1 1 1 1 1 1 1 1</td>
<td>1</td>
</tr>
</tbody>
</table>

**Table 2. Characteristics of clinical *Bartonella henselae* isolates from 5 case-patients, South Korea**


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etymologia revisited

**Bartonella henselae**

[bär’tə-nəl’ə henz’ ə-lä]

*Bartonella* is a genus of gram-negative bacteria named after Peruvian scientist Alberto Leonardo Barton. He identified a unique bacterium in 1905 during an outbreak among workers building a railway between Lima and La Oroya, a mining town in the Andes. The illness, usually fatal, was characterized by fever and severe anemia. Many of the sick were brought to Guadalupe Hospital in Lima, where Dr. Barton isolated the etiologic agent (which had been transmitted by sandflies) in patients’ blood cells. It was later called *Bartonella bacilliformis*.

The species *B. henselae* was named after Diane Hensel, a technologist in the clinical microbiology laboratory, University Hospitals, Oklahoma City, who in 1985 observed a *Campylobacter*-like organism in blood cultures of HIV-infected patients. The organism was first named *Rochalimaea henselae* and then *B. henselae*, when sequencing showed identity with that genus.


https://wwwnc.cdc.gov/eid/article/14/6/08-0980_article
Antimicrobial-Resistant Bacteria in Infected Wounds, Ghana, 2014

Hauke Janssen, Iryna Janssen, Paul Cooper, Clemens Kainyah, Theresia Pellio, Michael Quintel, Mathieu Monnheimer, Uwe Groß, Marco H. Schulze

Wound infections are an emerging medical problem worldwide, frequently neglected in under-resourced countries. Bacterial culture and antimicrobial drug resistance testing of infected wounds in patients in a rural hospital in Ghana identified no methicillin-resistant *Staphylococcus aureus* or carbapenem-resistant *Enterobacteriaceae* but identified high combined resistance of *Enterobacteriaceae* against third-generation cephalosporins and fluoroquinolones.

Bacteriologic investigation of clinical specimens is an essential tool for active surveillance of antimicrobial drug resistance. Knowledge of causative bacterial species and their resistance profile enables targeted antimicrobial therapy, limits ineffective antimicrobial therapy, and avoids in part unnecessary antimicrobial pressure to noninvolved bacterial pathogens (1). Available antimicrobial resistance data will sensitize clinicians and policy makers and are a prerequisite for updating national treatment guidelines (1, 2). These data contribute to prevention and control of antimicrobial drug resistance (1).

Wound infections are an emerging medical problem worldwide; the economic burden and morbidity and mortality rates are huge (3, 4). Because of the frequent polymicrobial nature of infected wounds, bacteriologic investigations are demanding and frequently neglected in sub-Saharan Africa countries (5).

The Study

Since 2000, the Institute for Medical Microbiology of the University Medical Center Goettingen, Goettingen, Germany, has assisted the running of the bacteriology laboratory in St. Martin de Porres Hospital in Eikwe, Ghana (2). Eikwe is a rural coastal village in the Western Region of Ghana; its mission hospital has an admission capacity of 200 beds and serves 380,000 persons.

During March–July 2014, we conducted a prospective study at St. Martin de Porres Hospital, performing bacteriologic investigations of infected wounds of inpatients and outpatients during routine working hours (Monday–Friday, 8 AM–4 PM). The hospital administration (the local ethics review panel) authorized the study. Patients from whom wound swab samples were investigated provided consent to be included in the study.

Medical doctors diagnosed wound infections clinically, according to the classic signs of inflammation. After wounds were carefully cleaned with sterile gauze moistened with a sterile solution of 0.9% sodium chloride, samples were collected from the wound ground and edge on sterile cotton swabs and immediately transported to the bacteriology laboratory in Amies transport medium (Copan, Brescia, Italy). The samples were inoculated onto MacConkey agar and 7% sheep blood agar (Tulip Diagnostics, Goa, India) and thereafter incubated aerobically at 35°C. Both plates were read after 24 and 48 hours. Gram staining was performed to ensure wound specimen quality and to check for bacteria, neutrophils, and epithelial cells.

Bacterial isolates were initially identified (to genus level) by colony morphology, Gram staining, catalase reaction, oxidase reaction, coagulase reaction, indole reaction, and growth on Kligler iron agar, as described by Cheesbrough (6). Bacterial isolates were stored in microbanks at −20°C. Species identification was completed (to species level) at the Institute for Medical Microbiology in Goettingen, Germany, by using MALDI Biotyper 3.0 (Bruker Daltonics, Bremen, Germany).

According to locally available resources, antimicrobial resistance testing was performed through disk diffusion, which guided the treatment of the wound infections. Antimicrobial resistance testing was repeated with VITEK 2 (bioMérieux, Marcy-l’Étoile, France) at the Institute for Medical Microbiology by using AST-P632, AST-P586, AST-N214, and AST-N248 cards with respect to bacterial species and according to the breakpoint tables for interpretation.

1Preliminary results from this study were presented at the Annual Meeting of the German Society of Tropical Medicine and International Health; October 7–8, 2016; Bonn, Germany; and at the 69th Annual Meeting of the German Society for Hygiene and Microbiology; March 5–8, 2017; Wuerzburg, Germany.

2These authors contributed equally to this article.
of MICs in EUCAST version 4.0 (7). Quality control was performed with the reference strains *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 29213.

Of the 67 wound swab samples, 39 (58.2%) were from female patients. The mean age of the 67 patients was 40.1 ± 20.8 years (range 1–90 years, median 39 years). Of the 67 samples, collection sites were upper extremity for 4 (6.0%), trunk/head for 15 (22.4%), lower extremity for 39 (58.2%), and laparotomy site for 9 (13.4%) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/17-1506-Techapp1.pdf). A hospital-acquired wound infection was diagnosed for 21 (31.3%) patients. The predominant bacteria in polymicrobial infections were *Enterobacteriaceae* and nonfermenters (online Technical Appendix Table 2). Of the 67 samples, infection was monomicrobial in 17 (25.4%) and polymicrobial in 50 (74.6%). The most frequently detected bacterium in monomicrobial and polymicrobial infections was *S. aureus*. The predominant bacteria in polymicrobial infections were *Enterobacteriaceae* and nonfermenters (online Technical Appendix Table 3). Results of VITEK 2 antimicrobial resistance testing of the most frequently found bacterial species are shown in Table 1.

The spectrum of isolated bacteria is comparable to that reported by other studies from sub-Saharan Africa countries, such as Nigeria (9), Tanzania (3), and Rwanda (10). Frequently, studies describe detected pathogens at the genus level only (3,10). Concerning the proportion of gram-positive to gram-negative pathogens, we isolated slightly more gram-positive pathogens than others (3,9–11).

One of the most common bacteria found in wound infections is *S. aureus* (3,5,10–12), which was most frequently identified in our study (online Technical Appendix Table 2); however, we detected no methicillin-resistant *S. aureus* (MRSA). In contrast, studies from urban areas in sub-Saharan Africa countries found MRSA rates of >80% (10,12). Urban areas are centers of specialized healthcare, where many patients who may already have a long medical history are referred. Such referrals predispose urban patients, staff, and others to more MRSA colonization and infection than experienced by those in rural areas (13). The hospital in Eikwe is a general hospital; the villagers are mainly fishermen, and there are no big animal farms in the area. Predisposition to MRSA in this area may be low.

We found no carbapenem resistance in *Enterobacteriaceae* (Table 1). Of great concern were the high rates of resistance of *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* complex against third-generation cephalosporins, fluoroquinolones, or both (Table 2), as have been found in other studies from urban areas (3,5,10). The

The interpretation of the CAZ MIC for *A. baumannii* complex followed the recommendations of the Clinical and Laboratory Standards Institute performance standards for antimicrobial susceptibility testing (8).

### Table 1. Percentages of antimicrobial drug resistance in selected bacterial species in wound infections, Ghana, 2014*

<table>
<thead>
<tr>
<th>Drug</th>
<th><em>Staphylococcus aureus, n = 31</em></th>
<th><em>Enterococcus faecalis, n = 21</em></th>
<th><em>Proteus mirabilis, n = 20</em></th>
<th><em>Escherichia coli, n = 19</em></th>
<th><em>Klebsiella pneumoniae, n = 13</em></th>
<th><em>Enterobacter cloacae complex, n = 10</em></th>
<th><em>Pseudomonas aeruginosa, n = 20</em></th>
<th><em>Acinetobacter baumannii complex, n = 8</em></th>
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<tr>
<td>PEN</td>
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*Antimicrobial susceptibility testing was performed with VITEK 2 (bioMérieux, Marcy-l'Étoile, France) according to the EUCAST breakpoint tables for interpretation of MICs, version 4.0, 2014 (7). Blank cells indicate no testing performed. AMI, amikacin; AMP, ampicillin; CAZ, cefazidime; CIP, ciprofloxacin; CLI, clindamycin; CTX, cefotaxime; CXM, cefuroxime; ERY, erythromycin; FOX, fosfomycin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; OXA, oxacillin; PEN, penicillin; RIF, rifampin; SAM, ampicillin/sulbactam; SXT, trimethoprim/sulfamethoxazole; TET, tetracycline; TZIP, piperacillin/tazobactam; VAN, vancomycin.

†The interpretation of the CAZ MIC for *A. baumannii* complex followed the recommendations of the Clinical and Laboratory Standards Institute performance standards for antimicrobial susceptibility testing (8).
indiscriminate use of antimicrobial drugs contributes to this factor (14). Officially, selling antibiotics without prescription is not allowed in Ghana; however, almost every oral antimicrobial drug is available over the counter without any prescription. Eikwe is no exception, although the spectrum of available antimicrobial drugs may be smaller there than in cities. Development of antimicrobial drug resistance may also be enhanced by circulation of counterfeit drugs (15).

Resistance of *E. coli* and *K. pneumoniae* against third-generation cephalosporins probably occurs through production of extended spectrum β-lactamase; in *E. cloacae* complex, it is probably through AmpC–β-lactamase. However, this statement is only an assumption because we did not perform molecular analyses.

In Eikwe, rain falls throughout the year and humidity is almost constant at 70%–90% despite 2 rainfall peaks (May–June and October–November). The effect of seasonality on the incidence of wound infections and the frequency of infection with gram-negative bacteria may not be as pronounced as that found in other studies from sub-Saharan Africa countries with high variations in humidity (9). However, because we analyzed only swab samples collected during March–July, the effect of seasonality is difficult to evaluate.

**Conclusions**

Antimicrobial drug resistance among gram-negative organisms seems to be widespread in Ghana, even among community-onset infections in rural, resource-limited settings, although MRSA was surprisingly absent. Future research efforts should focus on the transmission dynamics and prevention of gram-negative antimicrobial resistance in those settings. Microbiological investigation of the worldwide problem of wound infections should be encouraged in areas of limited resources and might provide a valuable contribution to the surveillance of increasing antimicrobial resistance, especially in *Enterobacteriaceae*, and for the treatment of affected patients.

**Acknowledgments**

We thank the patients in Ghana for their participation in this study. We also thank the staff from St. Martin de Porres Hospital and the staff from the Institute for Medical Microbiology of the University Medical Center Goettingen for their commitment to perform the study.

**About the Author**

Dr. Janssen is an anesthesiologist who during the study worked as a volunteer at St. Martin de Porres Hospital. He supported the medical personnel in the operating theater and trained them in anesthesiology, resuscitation, and general medicine.

**References**


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Follow the EID journal on Twitter and get the most current information from Emerging Infectious Diseases.
Seven foodborne norovirus outbreaks attributable to the GII.P17-GII.17 strain were reported across Japan in 2017, causing illness in a total of 2,094 persons. Nori (dried shredded seaweed) was implicated in all outbreaks and tested positive for norovirus. Our data highlight the stability of norovirus in dehydrated food products.

Seven foodborne norovirus outbreaks were reported in 4 remote areas across Japan during January–February 2017, causing illness in 2,094 persons (Figure). In all outbreaks, norovirus GII.P17-GII.17 was detected in stool specimens of patients. Food survey results indicated that dried shredded seaweed (nori) was served before all outbreaks. Norovirus was also detected in nori-containing dishes. An investigation revealed that the nori served in each instance was manufactured by the same food processing company.

The Study

Outbreak 1 was reported on January 26 in 4 kindergartens, 6 elementary schools, and 5 junior high schools in the prefecture of Wakayama, Japan (1). A total of 1,943 children and 119 school staff members had eaten lunch the day before; of these, 678 (34.9%) children and 85 (71.4%) school staff members had acute gastroenteritis. Norovirus was also detected in the stool specimens from 10 of the 27 food handlers in the central kitchen who had eaten the same lunch. Food survey results indicated that boiled vegetables mixed with shredded nori was responsible for this outbreak.

Outbreak 2 was reported on February 17 in 7 elementary schools in the city of Tachikawa, part of metropolitan Tokyo, Japan (1). A total of 1,084 (35.2%) of the 3,078 persons who ate the lunch served the day before had gastrointestinal symptoms. The lunch included shredded nori as a topping on cooked rice.

Outbreaks 3 and 4 were reported in separate, self-catered school lunch settings on February 22 and 25 in the city of Kodaira, also part of metropolitan Tokyo (1). The numbers of patients with gastrointestinal symptoms were 26 (5.6% attack rate) in outbreak 3 and 81 (12.6% attack rate) in outbreak 4. Shredded nori was served as a topping on cooked rice in both outbreaks.

Outbreak 5 was reported in Western Tama, also in metropolitan Tokyo, on February 27. A central kitchen served school lunch to 19 persons, and 2 of them (10.5%) had gastrointestinal symptoms. Shredded nori was served with boiled vegetables.

Outbreak 6 was reported on March 9. After conducting retrospective surveillance, the Kurume city government in Fukuoka prefecture announced that a foodborne outbreak occurred in a business office on January 25 that was attributable to a nori product with the same expiration date as the nori implicated in outbreaks 1–5. A total of 39 (92%) of the 42 employees who had eaten at the office’s café had gastrointestinal symptoms. Shredded nori had been served as a salad topping.

Outbreak 7, announced by the Osaka prefectural government, caused illness in 99 persons during February 18–24, including 4 food handlers who consumed a bento box (a single-portion take-out or home-packed meal common in Japanese cuisine). A bento shop provided 228 meal boxes during this period that contained nori product from company B. Those who had eaten from bento boxes without nori also had gastrointestinal symptoms.

We detected norovirus in nori-containing food in outbreak 1 by using the PANSORBIN-trap method (2).
Norovirus-Contaminated Nori, Japan

Tokyo Metropolitan Institute of Public Health tested norovirus from food from outbreaks 2–5 by using the A3T method, by which nori was incubated with the *Proteus vulgaris* NBRC3045 strain (3). Among 21 nori samples, including those from the merchandise in stock of the wholesaler and those that remained in the kitchen from outbreaks 2–5 in Tokyo, 7 samples were positive for norovirus GII.17 by nested reverse transcription PCR (4–6). These data, along with the food survey results, strongly suggest that nori was responsible for the food-borne norovirus outbreaks.

The amount of shredded nori served as a topping on cooked rice was 0.5–1.0 g per dish. The shredded nori contained 360–2,900 copies/g of norovirus genome, a measure that did not take into account the recovery rate of the virus particles from the food (4). In outbreak 7, food handlers shared and reused plastic gloves, which increased the risk for norovirus contamination across food.

Nori sheets were originally produced by company C, and food processing company B shredded them to 2-mm width and packed them with desiccants (Figure). Approximately 800 packages were produced, shipped to company A on December 10 and 27, 2016, and sold under the company A brand with the same expiration date. Another batch was produced by company B in December 2016 and sold under its own brand with an expiration date of May 31, 2017. These packages were stored at an ambient temperature.

On February 27, 2017, the Osaka city government announced that norovirus GII.17 was detected from 8 of 25 environmental wipe samples at company B. These samples were subjected to ultracentrifugation to precipitate norovirus particles before nucleic acid extraction. Samples collected from the nori sheet shredding equipment, a telephone handle, and the toilet were positive for norovirus. At company B, an employee who was responsible for the

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**Figure.** Overview of foodborne norovirus outbreaks associated with dried shredded nori during the 2016–17 endemic season, Japan. Production and distribution of dried, shredded nori products and the 7 norovirus outbreaks are outlined. Solid and dashed lines indicate the production and distribution of 2 distinct lots. The asterisk indicates detection of norovirus in food, shredded nori, or both. Inset map shows geographic locations of outbreaks and manufacturers.
recovery of norovirus genomic RNA from food is not during manufacturing. From a technical standpoint, the shredded nori product that was most likely contaminated breaks in 4 remote regions of Japan were attributable to a -

Our study demonstrated that 7 foodborne norovirus out-

Conclusions

Few dry food–associated foodborne outbreaks with norovirus have been reported previously. In 2014, a large norovirus outbreak involving 1,271 persons was reported in which bread was contaminated with norovirus. The contaminated bread was served at school lunch within 2 days after production (9). In contrast, in these nori-related outbreaks, the traceback of implicated nori product revealed that the norovirus infectivity remained for >2 months at ambient temperature under dry conditions. However, the percentage of persons with gastrointestinal symptoms gradually decreased from the date of nori production, suggesting a decline in norovirus infectivity over time under dry conditions.

Acknowledgments

We are grateful to Mamoru Noda for information on the PANSORBIN-trap method and to Jan Vinjé for valuable comments on the manuscript.

The Japan Agency for Medical Research and Development supported this work.

Author contributions: Detection of norovirus and genetic analysis were managed by each institute of public health. Y.M. and H.S. designed specific primers for GII.P17-GII.17. K.N., H.K., and N.S. conducted the phylogenetic tree analysis. N.S. and J.K. wrote the manuscript. All authors commented on the manuscript.

About the Author

Dr. Sakon is a senior research scientist whose primary research interests include public health and viral infections of the gastrointestinal tract.

Table. Genetic analysis of norovirus GII.P17-GII.17 isolates detected in clinical specimens from outbreaks during the 2016–17 endemic season, Japan*

<table>
<thead>
<tr>
<th>Outbreak or strain</th>
<th>GenBank accession nos.</th>
<th>Isolation date</th>
<th>Identity with outbreak 1 strain, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RdRp, 1,530 nt</td>
</tr>
<tr>
<td>Outbreak 1</td>
<td>LC318755–LC318758</td>
<td>Jan 2017</td>
<td>NA</td>
</tr>
<tr>
<td>Outbreak 2</td>
<td>LC258403†</td>
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<td>100</td>
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<tr>
<td>Outbreak 6</td>
<td>LC311767–LC311773</td>
<td>Jan 2017</td>
<td>100</td>
</tr>
<tr>
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</tr>
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<tr>
<td>Outbreak D:16–311</td>
<td>LC318747</td>
<td>Dec 2016</td>
<td>99.9</td>
</tr>
<tr>
<td>Outbreak D:16–315</td>
<td>LC318748</td>
<td>Dec 2016</td>
<td>99.8</td>
</tr>
<tr>
<td>GII.17 Kawasaki308</td>
<td>LC037415</td>
<td>Feb 2015</td>
<td>99.6</td>
</tr>
</tbody>
</table>

*NA, not applicable; ND, not done.
†This isolate was registered as a representative strain of outbreaks 2–5 because all the isolates from outbreaks 2–5 showed 100% sequence identity.

Most foodborne outbreaks in Japan are attributable to norovirus GII.P17-GII.17, a novel variant that emerged in Asia during 2014–2015 (7,8). The genome sequence spanning the N terminus of the major capsid protein (VP1) gene, a short 302-nt region that is routinely sequenced, was identical among the GII.P17-GII.17 strains isolated in Japan during the 2016–17 season. Therefore, we sequenced and analyzed 2 virus genes, RNA-dependent RNA polymerase (RdRp) (1,530 nt) and VP1 (1,620 nt). For all 7 outbreaks, the sequence identities of these 2 genes derived from patient stool specimens were 100% identical (Table). As a comparison, we analyzed norovirus GII.P17-GII.17 strains detected from 4 independent outbreaks unrelated to nori products during the same season in Osaka (Table). The sequences of 2 genes among these strains were not identical with those related to nori-associated outbreaks, and phylogenetic tree analyses of the RdRp gene showed that sequences associated with the nori-related norovirus outbreaks formed a distinct cluster from the outbreaks unrelated to nori (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/5/17-1733-Techapp1.pdf). These results and epidemiologic link indicated that all 7 outbreaks were attributable to GII.P17-GII.17–contaminated shredded nori.

Conclusions

Our study demonstrated that 7 foodborne norovirus outbreaks in 4 remote regions of Japan were attributable to a shredded nori product that was most likely contaminated during manufacturing. From a technical standpoint, the recovery of norovirus genomic RNA from food is not efficient, so identifying the contaminated food can be difficult; however, the PANSORBIN-trap and A3T methods were shown to be useful for this purpose. Sequence analysis of the RdRp and VP1 regions of the norovirus genome enabled us to distinguish nori-related outbreaks involving norovirus GII.P17-GII.17 from other outbreaks involving the same genotype.

shredding step had gastrointestinal symptoms before the production of the nori in question in late December 2016 but nevertheless continued working. No outbreak had been reported after the products were recalled, and the business of company B was suspended. Investigators suspected that the nori was contaminated with norovirus during the shredding process at company B.

Few dry food–associated foodborne outbreaks with norovirus have been reported previously. In 2014, a large norovirus outbreak involving 1,271 persons was reported in which bread was contaminated with norovirus. The contaminated bread was served at school lunch within 2 days after production (9). In contrast, in these nori-related outbreaks, the traceback of implicated nori product revealed that the norovirus infectivity remained for >2 months at ambient temperature under dry conditions. However, the percentage of persons with gastrointestinal symptoms gradually decreased from the date of nori production, suggesting a decline in norovirus infectivity over time under dry conditions.

ACKNOWLEDGMENTS

We are grateful to Mamoru Noda for information on the PANSORBIN-trap method and to Jan Vinjé for valuable comments on the manuscript.

The Japan Agency for Medical Research and Development supported this work.

Author contributions: Detection of norovirus and genetic analysis were managed by each institute of public health. Y.M. and H.S. designed specific primers for GII.P17-GII.17. K.N., H.K., and N.S. conducted the phylogenetic tree analysis. N.S. and J.K. wrote the manuscript. All authors commented on the manuscript.

About the Author

Dr. Sakon is a senior research scientist whose primary research interests include public health and viral infections of the gastrointestinal tract.
References


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April 2016: Food Safety

- Determinants and Drivers of Infectious Disease Threat Events in Europe
- Shiga Toxin–Producing Escherichia coli O157, England and Wales, 1983–2012
- Nosocomial Co-Transmission of Avian Influenza A(H7N9) and A(H1N1)pdm09 Viruses between 2 Patients with Hematologic Disorders
- Quantifying Transmission of Clostridium difficile within and outside Healthcare Settings
- Microevolution of Monophasic Salmonella Typhimurium during Epidemic, United Kingdom, 2005–2010
- Molecular Typing and Epidemiology of Human Listeriosis Cases, Denmark, 2002–2012
- Limited Dissemination of Extended-Spectrum β-Lactamase– and Plasmid-Encoded AmpC–Producing Escherichia coli from Food and Farm Animals, Sweden
- Transmission of Middle East Respiratory Syndrome Coronavirus Infections in Healthcare Settings, Abu Dhabi
- Lassa Virus Seroprevalence in Sibirilia Commune, Bougouni District, Southern Mali
- Neisseria meningitidis Serogroup X in Sub-Saharan Africa Post-Ebola Syndrome, Sierra Leone
- Evaluation of Viremia Frequencies of a Novel Human Pegivirus by Using Bioinformatic Screening and PCR
- Shiga Toxin 1–Producing Shigella sonnei Infections, California, United States, 2014–2015
- Adenovirus Type 7 Pneumonia in Children Who Died from Measles-Associated Pneumonia, Hanoi, Vietnam, 2014
- Elevated Toxoplasma gondii Infection Rates for Retinas from Eye Banks, Southern Brazil
- Arenavirus Diversity and Phylogeography of Mastomys natalensis Rodents, Nigeria
- Cross-Neutralization between Human and African Bat Mumps Viruses

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Bartonella henselae DNA in Seronegative Patients with Cat-Scratch Disease

Masashi Yanagihara, Hidehiro Tsuneoka, Ayano Tanimoto, Ken-ichiro Otsuyama, Jun Nishikawa, Tomohiro Matsu, Junzo Nojima, Kyosih Ichihara

Author affiliation: Yamaguchi University, Ube, Japan

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We used real-time PCR to detect Bartonella henselae DNA in 7.9% (5/63) of blood specimens from seronegative patients in Japan suspected of having cat-scratch disease. The combined use of serologic tests and real-time PCR to analyze blood specimens is recommended for the prompt, noninvasive laboratory diagnosis of cat-scratch disease.

Cat-scratch disease (CSD) is a worldwide zoonosis caused by Bartonella henselae (1). Its clinical manifestations vary from typical CSD with regional lymphadenopathy to atypical or systemic CSD, including prolonged fever without lymphadenopathy. Because isolation of B. henselae by culture is difficult (2), detection of B. henselae DNA in lymph nodes by PCR and serologic testing using indirect fluorescence antibody (IFA) assay is widely used for laboratory diagnosis of CSD (2–4). Isolation of B. henselae DNA from blood specimens of immunocompetent CSD patients has been sporadically described, suggesting that it might be useful, especially for cases in which lymphadenectomy or biopsy is not feasible or serologic results are equivocal (5–9). However, the usefulness of serologic testing, coupled with detection of B. henselae DNA from blood specimens, is not well described. We report the clinical utility of the combined use of IFA and real-time PCR to analyze blood specimens for noninvasive screening of CSD.

During April 2009–May 2014, eighty immunocompetent patients (73 children, 7 adults) in Japan who were suspected of having CSD because of fever with or without lymphadenopathy, and a history of contact with cats or dogs were referred to us for serologic and molecular diagnosis of CSD. We conducted serologic testing using IFA (3,4); diagnosis was based on elevated titer of IgM (≥20) or IgG (≥256). The sensitivity and specificity of our IFA were 69% and 100%, respectively (4). Real-time PCR (rPCR) detected specific B. henselae virB4 DNA from blood specimens as reported previously (9). In brief, we extracted DNA from peripheral blood using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). The 20-µL PCR mixture contained 10 µL × 2 LightCycler 480 Probes Master Mix (Roche Diagnostics, Mannheim, Germany), 0.4 µmol/L of each primer (forward: 5’-AGCGAAGAAAACACAATCTGAA-3'; reverse: 5’-TCCATAGTTTTCAATCTTCTT-3’), 0.1 µmol/L Universal ProbeLibrary probe no. 135 (Roche Diagnostics), and 5 µL DNA. We conducted the reaction using a LightCycler 480 instrument (Roche Diagnostics) under the following conditions: denaturation at 95°C for 5 min and 45 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. We determined crossing point (Cp) values using the second derivative maximum method and analyzed all samples in triplicate.

This assay detected all 56 specimens evaluated as B. henselae (10), but other Bartonella species were not identified (9). The lower quantitative detection limit of this assay was 4.6 DNA copies/reaction using serial dilutions of plasmid DNA.

Of the 80 patients with suspected CSD, 17 (21.3%) were serologically positive for B. henselae by IFA. B. henselae DNA was amplified from the peripheral blood of 11 (13.8%) patients by rPCR. Six patients were positive by both IFA and rPCR. CSD was diagnosed in 22 (27.5%) of the 80 patients (Table). Cp values of the IFA-positive and -negative patients did not differ.

Despite the high specificity, IFA lacks sensitivity (4). This study showed that CSD detection sensitivity increased from 21.3% (17/80) using IFA alone to 27.5% (22/80) with combined use of IFA and rPCR on blood specimens. We attribute this increase to the detection of B. henselae DNA in 5 patients with seronegative results. Four patients (nos. 6, 8, 10, and 17) exhibited typical CSD with fever and regional lymphadenopathy, and 1 (no. 3) had fever of unknown origin without lymphadenopathy (Table). After laboratory diagnosis, these patients were treated with macrolides to reduce fever. These observations suggested that these patients were in the initial stages of the illness (before a significant rise of antibodies to B. henselae) or that the patients’ immune responses were insufficient to produce antibodies to B. henselae (5).

We detected B. henselae DNA in blood specimens of 35.3% (6/17) of the seropositive patients in our study, whereas previous studies detected DNA in 19.2% (5/26) of blood specimens from seropositive patients (5) and 16.7% (3/18) of serum specimens from patients proven to have CSD (6). rPCR using blood specimens was not sensitive enough when used alone because B. henselae DNA is not present in the bloodstream in all patients. The time points at which B. henselae DNA can be detected in blood specimens are still unknown. A previous report described this time point as 3 weeks after onset of lymphadenopathy (7), whereas another study considered it to be 3–4 months after infection (8). Most specimens used in our study were
collected within 3 weeks after symptom onset. Regardless of the days after onset, rPCR testing of blood specimens should be performed actively because patients may experience bacteremia or the shedding of bacterial breakdown products into the bloodstream.

In conclusion, our study showed that rPCR testing of blood specimens can detect *B. henselae* DNA in patients with seronegative results. The combined use of IFA and rPCR on blood specimens is useful for the noninvasive screening of CSD.

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### About the Author

Dr. Yanagihara is an assistant professor in the Department of Laboratory Sciences, Faculty of Health Sciences, Yamaguchi University Graduate School of Medicine. His primary research interest is *B. henselae* infection.

### References


### Table. Description of patients with cat-scratch disease and results of IFA and rPCR of blood specimens, Japan, April 2009–May 2014*

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Patient age, y/sex</th>
<th>Fever</th>
<th>Regional lymphadenopathy</th>
<th>History of contact with animal</th>
<th>IFA titer</th>
<th>Cp of rPCR, mean ± SD</th>
<th>Complication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15/M</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>4/F</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>6/F</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>13/M</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>14/F</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td></td>
<td>&lt;10</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>10/M</td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>256</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>8/F</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
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</tr>
<tr>
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<td>10/M</td>
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</tr>
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<td></td>
<td></td>
<td>10</td>
<td>&lt;64</td>
<td></td>
</tr>
<tr>
<td>11</td>
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<td></td>
<td></td>
<td></td>
<td>40</td>
<td>256</td>
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</tr>
<tr>
<td>12</td>
<td>15/M</td>
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<td></td>
<td>&lt;10</td>
<td>512</td>
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</tr>
<tr>
<td>13</td>
<td>6/F</td>
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<td>Cat, dog</td>
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<td>Neuroretinitis</td>
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<td>14</td>
<td>10/M</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>15</td>
<td>12/F</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>16</td>
<td>13/M</td>
<td></td>
<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td>Neuroretinitis</td>
</tr>
<tr>
<td>17</td>
<td>9/F</td>
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<td>Neuroretinitis</td>
</tr>
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<tr>
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<td></td>
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<td>512</td>
<td>Neuroretinitis</td>
</tr>
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<td>56/F</td>
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<td></td>
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<td>80</td>
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<td>Neuroretinitis</td>
</tr>
<tr>
<td>21</td>
<td>35/F</td>
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<td></td>
<td></td>
<td>&lt;10</td>
<td>512</td>
<td>Neuroretinitis</td>
</tr>
<tr>
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<td>7/M</td>
<td></td>
<td></td>
<td></td>
<td>40</td>
<td>1,024</td>
<td></td>
</tr>
</tbody>
</table>

*Blank cells indicate no complications. Cp, crossing point value; FUO, fever of unknown origin; IFA, indirect fluorescent antibody test; NA, no amplification by rPCR; rPCR, real-time PCR; +, positive; −, negative.
Middle East Respiratory Syndrome Coronavirus Antibodies in Dromedary Camels, Bangladesh, 2015


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Dromedary camels are bred domestically and imported into Bangladesh. In 2015, of 55 camels tested for Middle East respiratory syndrome coronavirus in Dhaka, 17 (31%) were seropositive, including 1 bred locally. None were PCR positive. The potential for infected camels in urban markets could have public health implications and warrants further investigation.

Middle East respiratory syndrome coronavirus (MERS-CoV), discovered in 2012, can cause fatal respiratory disease in humans. Although MERS-CoV might have originated in bats, dromedary camels (Camelus dromedarius) are a natural host and likely source of human MERS-CoV infection (1,2). Camel trade is a major driver of MERS-CoV movement between Africa and the Arabian Peninsula (3), where most human cases have occurred. Rajasthan, India, is a large breeding center for dromedaries, some of which are exported to Pakistan and Bangladesh. Seropositive dromedaries have been identified in Pakistan, but little is known about MERS-CoV in other parts of South Asia (4). In Bangladesh, camels are bred on farms and imported from India for sale in seasonal markets for ritual slaughter during religious festivals. Imported camels go directly to urban markets to be sold by traders and are a separate enterprise from farmed camels.

During the September–October 2015 festival of Eid-ul-Adha, we collected and tested for coronaviruses the specimens of 36 dromedary camels at an urban farm and 19 camels and 18 fat-tailed sheep at an urban maket in the capital city of Dhaka (Table; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/17-1192-Techapp1.pdf). The testing was conducted as part of the US Agency for International Development’s PREDICT program, which conducts surveillance in humans and animals for novel and select known zoonotic viruses, including MERS-CoV. We obtained information for each camel’s origin and age from market registries or breeders’ records. We also assessed and recorded the sex and apparent health status of each camel at specimen collection, at which time we collected blood, 2 nasal swab specimens, and 2 rectal swab specimens from each animal. We placed 1 set of each swab in lysis buffer (NucliSens; bioMérieux, Marcy-l’Étoile, France) and 1 in viral transport medium. We separated and froze serum samples. We extracted total nucleic acid by using EasyMag (bioMérieux) and performed cDNA synthesis by using SuperScript III first-strand synthesis supermix (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. We performed pancoronavirus PCR targeting the RdRp gene (5) and MERS-CoV real-time PCR targeting the upstream envelope protein gene and nucleocapsid protein genes N2 and N3 (6). We screened serum samples by using a MERS-CoV ELISA (7) and confirmed the results by using a MERS-CoV pseudoparticle neutralization test (8).

Of the 36 camels on the farm, 24 were born there. The remaining 12 and all 19 market camels were imported from India (Table). All specimens tested negative for coronaviruses, including MERS-CoV, by PCR. ELISA showed 98.6% specificity and sensitivity compared with the pseudoparticle neutralization test. We detected MERS-CoV antibodies in 31% (95% CI 19%–45%) of camels; adults had a higher seroprevalence (36% [95% CI 22%–52%]) than juveniles (9% [95% CI 0.2%–41%]). Imported camels had a significantly higher seroprevalence (52% [95% CI 33%–70%]) than domestically bred camels (4% [95% CI 0.1%–21%]). Among the 5 seropositive farm camels, 1 was a domestically bred adult, whereas the other 4 were adults from India. Camels in the market had a higher seroprevalence (63% [95% CI 38%–85%]) than those on the farm (14% [95% CI 5%–30%]). All sheep serum samples were negative for MERS-CoV antibodies.

The findings of a higher MERS-CoV seroprevalence in adult camels (9) and the seronegativity in sheep are consistent with other studies (8). Only adult camels were found in the market. The finding of an adult seropositive camel, born on the farm, suggests that it was infected locally. No records indicate intermingling between farmed camels and those in markets. The finding of only 1 seropositive camel originating in Bangladesh suggests that if infection or exposure occurred on the farm, either viral circulation was limited or other seropositive camels had since been sold or removed. Juveniles are more likely to be actively infected than adults, and the limited juvenile sample size might explain our lack of virus detection among them (9).
Our findings suggest transmission of MERS-CoV has occurred among camels in Bangladesh, extending the previously reported range of this virus (up to ≈1,900 km east of Pakistan). Exactly where or when imported camels became infected is unclear. To date, no human cases of MERS-CoV have been reported in South Asia. The possibility of having MERS-CoV–infected camels in Dhaka, a populous city with ≈18 million persons, presents a potential risk for human outbreaks. Insufficient surveillance, behavioral differences in human–camel interactions compared with Middle Eastern societies, or differences in virus strains or human susceptibility might explain the lack of observed cases. Improved surveillance of camels along camel trade routes, camel herds in Dhaka, and persons who have close contact with camels will help assess the transboundary movement and the risk for zoonotic transmission in Bangladesh. Given the ubiquity of MERS-CoV in dromedary camels, the predictable seasonal movement of camels into Dhaka, and a higher incidence of infection in persons with frequent contact with camels (10), targeted public health messaging that promotes handwashing after contact with camels and avoidance of exposure to camel excreta might help reduce the risk for zoonotic MERS-CoV transmission.

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**Table.** Selected characteristics and MERS-CoV laboratory results for dromedary camels, Bangladesh, 2015*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) camels</th>
<th>rPCR (%)</th>
<th>ELISA (%; 95% CI)</th>
<th>ppNT (%; 95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All camels</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Location</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Farm</td>
<td>36 (56)</td>
<td>0</td>
<td>6 (17, 6–33)</td>
<td>5 (14, 5–29)</td>
</tr>
<tr>
<td>Market</td>
<td>19 (35)</td>
<td>0</td>
<td>11 (58, 34–80)</td>
<td>12 (63, 38–84)</td>
</tr>
<tr>
<td><strong>Origin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Imported</td>
<td>31 (56)</td>
<td>0</td>
<td>16 (52, 33–70)</td>
<td>16 (62, 33–70)</td>
</tr>
<tr>
<td>Bangladesh</td>
<td>24 (44)</td>
<td>0</td>
<td>1 (4, 1–21)</td>
<td>1 (4, 0–21)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>29 (53)</td>
<td>0</td>
<td>6 (21, 8–40)</td>
<td>7 (24, 10–44)</td>
</tr>
<tr>
<td>F</td>
<td>26 (47)</td>
<td>0</td>
<td>11 (42, 23–63)</td>
<td>10 (38, 20–59)</td>
</tr>
<tr>
<td><strong>Age group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult, ≥2 y</td>
<td>44 (80)</td>
<td>0</td>
<td>16 (36, 22–52)</td>
<td>16 (36, 22–52)</td>
</tr>
<tr>
<td>Juvenile, &lt;2 y</td>
<td>11 (20)</td>
<td>0</td>
<td>1 (9, 1–41)</td>
<td>1 (9, 0–41)</td>
</tr>
<tr>
<td><strong>Body condition</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>19 (35)</td>
<td>0</td>
<td>11 (58, 34–80)</td>
<td>12 (63, 38–84)</td>
</tr>
<tr>
<td>Fair</td>
<td>6 (11)</td>
<td>0</td>
<td>1 (16, 1–64)</td>
<td>0</td>
</tr>
<tr>
<td>Good</td>
<td>30 (55)</td>
<td>0</td>
<td>5 (17, 1–35)</td>
<td>5 (17, 06–35)</td>
</tr>
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<td><strong>Upper respiratory signs</strong></td>
<td></td>
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</tr>
<tr>
<td>Yes</td>
<td>2 (4)</td>
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<td>1 (50, 1–99)</td>
<td>0</td>
</tr>
<tr>
<td>No</td>
<td>53 (96)</td>
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<td>16 (30, 18–44)</td>
<td>17 (32, 20–46)</td>
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<td><strong>Age group</strong></td>
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<td>0</td>
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<td>5 (18, 6–37)</td>
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<td>1 (6, 1–27)</td>
<td>4 (22, 6–48)</td>
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<td>5 (28, 10–53)</td>
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<td><strong>Market camels</strong></td>
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<tr>
<td><strong>Age group</strong></td>
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<td>11 (58, 34–80)</td>
<td>12 (63, 38–84)</td>
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</tbody>
</table>

* MERS-CoV, Middle East respiratory syndrome coronavirus; ppNT, pseudoparticle neutralization test; rPCR, real-time PCR.
generous support of the American people through the US Agency for International Development Emerging Pandemic Threats PREDICT project. The contents are the responsibility of the authors and do not necessarily reflect the views of the US Agency for International Development or the US Government.

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References

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Borrelia miyamotoi sensu lato in Père David Deer and Haemaphysalis longicornis Ticks

Yi Yang, Zhangping Yang,1 Patrick Kelly, Jing Li, Yijun Ren, Chengming Wang1

Author affiliations: Yangzhou University College of Veterinary Medicine, Yangzhou, China (Y. Yang, J. Li, C. Wang); Jiangsu Co-innovation Center for the Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou (Y. Yang, J. Li, C. Wang); Yangzhou University College of Animal Science and Technology, Yangzhou (Z. Yang); International Corporation Laboratory of Agriculture and Agricultural Products Safety, Yangzhou University, Yangzhou (Z. Yang); Ross University School of Veterinary Medicine, Basseterre, St. Kitts and Nevis (P. Kelly); Dafeng Elk National Natural Reserve, Yancheng, China (Y. Ren); Auburn University, Auburn, Alabama, USA (C. Wang)

By sequence analysis of 16S rRNA, flaB, p66, and glpQ, we identified Borrelia miyamotoi in 1 of 4 Père David deer (n = 43) seropositive for Borrelia spp. and 1.2% (3/244) of Haemaphysalis longicornis ticks from Dafeng Elk National Natural Reserve, China. Future studies should assess Borrelia pathogenesis in deer.

Père David deer (Elaphurus davidianus) are extinct in the wild and found only in captivity, principally in China, England, and the United States. Just 5,000 animals remain, with 40% located in Dafeng Elk National Natural Reserve in China, which attracts >1 million tourists annually. Ticks are common in the Dafeng Elk National Natural Reserve (1), so we investigated the tickborne bacterial pathogens in Père David deer at this reserve.

The institutional animal care and use committee of Yangzhou University College of Veterinary Medicine (Yangzhou, China) (YZU-CVM#2015–076) approved this study. We took whole blood samples from 43 apparently healthy Père David deer (20 males, 23 females), separated out the plasma (1,800 × g for 10 min), and used the plasma to detect antibodies against bacterial pathogens with the

1These senior authors contributed equally to this article.
Figure. Neighbor-joining phylogenetic trees constructed with 16S rRNA, flaB, p66, and glpQ gene sequences of Borrelia spp. isolates collected from Père David deer (Elaphurus davidianus) and Haemaphysalis longicornis ticks, Dafeng Elk National Natural Reserve, China, and reference isolates. The isolates identified in this study (bold; GenBank accession nos. MF521973, MF541143, MG763228, MG763229) are most similar to B. miyamotii of the relapsing fever group. Numbers at branch nodes show bootstrap support (1,000 replicates). Scale bars indicate nucleotide substitutions per site.
SNAP 4Dx kit (IDEXX, Westbrook, ME, USA) (2) according to the manufacturer’s instructions. Further, ELISAs and Western blots using Borrelia miyamotoi GlpQ recombinant protein (RayBiotech, Norcross, GA, USA) and peroxidase-labeled rabbit anti-deer IgG (SeraCare, Milford, MA, USA) were performed as described previously (3) to detect GlpQ antibodies specific to B. miyamotoi.

We collected a convenience sample of Haemaphysalis longicornis ticks (n = 244) from elk in the Dafeng Elk National Natural Reserve during the summer of 2016 and stored the collection at −80°C. We used the High Pure PCR Template Preparation Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions to extract DNA from Père David deer whole blood samples and entire H. longicornis ticks. We used published PCR protocols targeting the 16S rRNA (4), flaB (5), and glpQ (6) genes and an in-house p66 PCR (forward primer 5′-CGATTTTCTATATTGGACACAT-3′, reverse primer 5′-GATATAGATTCTACAGGTATTG-CATAATC-3′) to screen blood samples and ticks for B. miyamotoi. We sequenced both strands of PCR products using BGI’s (Shanghai, China) services and aligned them using ClustalW in MEGA 7 (http://www.megasoftware.net) with the nucleotide sequences of 11 relapsing fever group borreliae and 7 Lyme disease group borreliae found in GenBank.

Four (9.3%; 1 male, 3 females) of the 43 deer were seropositive by SNAP 4Dx, demonstrating an immunodominance of antibodies against synthetic C6 peptide invariable region 6 of the pathogenic Borrelia genospecies, B. burgdorferi sensu stricto, B. garinii, and B. afzelii (7). Seropositivity was confirmed by GlpQ antibody ELISA and Western blot with GlpQ recombinant protein, indicating exposure to B. miyamotoi.

One of the seropositive female deer (2.3% of overall deer population) and 3 (1.2%) of the 244 ticks were positive for the 4 Borrelia genes tested (16S rRNA, flaB, glpQ, p66) by PCR. The sequences obtained from the PCR products showed the 4 animals had identical sets of Borrelia genes. The 16S rRNA, flaB, and p66 sequences were more similar to those of the relapsing fever group borreliae (16S rRNA 97.9%–99.3%, flaB 83.7%–88.9%, p66 72.4%–83.3%) than the Lyme disease group borreliae (16S rRNA 96.6%–97.2%, flaB 79.2%–80.9%, p66 66.1%–68.1%).

The B. miyamotoi glpQ gene sequence obtained from the deer and ticks also clustered with those of the relapsing fever group borreliae (81.1%–88.9%), and all analyzed gene sequences had greatest similarity with B. miyamotoi genes (16S rRNA 99.3% [576/580], flaB 88.9% [321/361], p66 83.3% [423/508], glpQ 88.9% [377/424]) (Figure).

B. miyamotoi is a member of the relapsing fever group first isolated in Japan and subsequently found in North America, Europe, and Russia (8). B. miyamotoi has not been reported in deer but can be pathogenic in humans, usually resulting in an acute febrile influenza-like illness but occasionally causing severe disease, including meningoencephalitis (9). Further studies are needed to determine the effects of B. miyamotoi infections in deer, especially because studies on Ixodes scapularis ticks in the United States have indicated that deer might be a Sylvatic reservoir (10).

I. persulcatus and I. pavlovskyi ticks are known to be infected with B. miyamotoi in Asia, whereas other Ixodes spp. ticks are vectors in the United States and Europe (9). Tick control in semi–free-ranging animals is challenging; the Père David deer we studied are commonly infested with ticks. The only tick species identified on Père David deer in Dafeng Elk National Natural Reserve was H. longicornis (1), which can reach high densities in the environment (summer 89.5 ± 17.1 ticks/10 m², winter 1.47 ± 0.35 ticks/10 m²) and cause anemia and even death in heavily infested animals. Our finding of B. miyamotoi in H. longicornis ticks adds to the list of organisms reported in this tick, primarily B. burgdorferi sensu lato and unclassified Borrelia spp.

In summary, we have shown that B. miyamotoi sensu lato occurs in Père David deer and H. longicornis ticks in Dafeng Elk National Natural Reserve. Further studies are needed on the pathogenicity of the organism in deer and the role of H. longicornis ticks in the epidemiology of infections in deer and humans.

This project was supported by the National High Technology Research and Development Program of China (2013AA102505-5), National Natural Science Foundation of China (31472067), Scientific Innovation Research of College Graduate Program in Jiangsu Province (KYZZ16_0496), the Excellent Doctoral Dissertation Scholarship of Yangzhou University, and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

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References
**Rickettsia asembonensis** Characterization by Multilocus Sequence Typing of Complete Genes, Peru

Steev Loyola, Carmen Flores-Mendoza, Armando Torre, Claudine Kocher, Melanie Melendrez, Alison Luce-Fedrow, Alice N. Maina, Allen L. Richards, Mariana Leguia

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DOI: https://doi.org/10.3201/eid2405.170323

While studying rickettsial infections in Peru, we detected *Rickettsia asembonensis* in fleas from domestic animals. We characterized 5 complete genomic regions (17kDa, gltA, ompA, ompB, and sca4) and conducted multilocus sequence typing and phylogenetic analyses. The molecular isolate from Peru is distinct from the original *R. asembonensis* strain from Kenya.
JWSW01000078.1) (10) indicates high identity at the nucleotide (99.8%–100.0%) and amino acid (99.6–100.0%) levels (Table). As expected, conserved genes (17kDa and gltA) showed fewer substitutions than variable genes (ompA, ompB, and sca4). The 17-kDa gene exhibited no mutations along its 480-nt open reading frame (ORF), whereas the gltA gene exhibited 3 mutations along its 1,314-nt ORF. Two mutations in gltA encoded silent changes, whereas the third encoded a conservative lysine-to-glutamic acid change at position 290. In the variable group, ompB exhibited no mutations along its 4,947-nt ORF; ompA and sca4 exhibited 5 each. ompA had 2 conservative changes (leucine-to-valine at position 1454 and valine-to-alanine at position 1627) and 2 nonconservative changes (tyrosine-to-aspartic acid at position 162 and arginine-to-glutamate at position 1280). sca4 had 1 conservative change (glutamine-to-histidine at position 608) and 3 nonconservative changes (leucine-to-proline at position 128, arginine-to-glutamic acid at position 754, and isoleucine-to-threonine at position 831). On the basis of these data, we conclude that the Peru molecular isolate is distinct from the original Kenya strain.

To further characterize the Peru isolate, we conducted phylogenetic analysis using the conserved gltA gene. Although reference sequences are available for multiple Rickettsiaceae, R. asembonensis sequences are limited in number and length (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/24/5/17-0323-Techapp1.pdf). Nevertheless, we constructed a phylogenetic tree using almost the complete gltA gene (1,068 [81%] nt of the ORF). As expected, the Peru isolate groups with RFLOs, including other R. asembonensis isolates and R. senegalensis (online Technical Appendix Figure, panel A). Construction of an additional tree using only 348 nt of gltA sequence available for an increased number of isolates (online Technical Appendix Table) enabled us to confirm placement and relationship with other strains from the Americas (online Technical Appendix Figure, panel B). This tree focuses exclusively on the transitional group and includes partial R. asembonensis references from Brazil, Colombia, and Costa Rica that were not available for inclusion in the 1,068-nt gltA tree. The Peru isolate clearly groups with other American isolates, and this subgroup is distinct from the original Kenya strain.

R. asembonensis is a new species (2) with potential as a ubiquitous human pathogen. Despite worldwide distribution, whether R. asembonensis and other RFLOs are pathogenic to humans, as is their closest relative R. felis, remains unknown. Complete genomic data, which are largely lacking from public repositories, are required to assess genetic diversity. Using next-generation sequencing, we generated complete sequences for 2 conserved (17 kDa and gltA) and 3 variable (ompA, ompB, and sca4) genes of an R. asembonensis molecular isolate from Peru. Although characterization of 1 isolate is not sufficient to evaluate strain diversity within Peru, much less among American strains, these sequences represent a major contribution toward the expansion of availability of much needed genomic information. Our multilocus sequence typing and phylogenetic analyses indicate that the Peru isolate is closer to American strains than to the original strain from Kenya. Characterization of additional isolates, derived from a variety of ectoparasites in which R. asembonensis has been detected, is needed to further validate our findings and to conduct in-depth diversity studies. In turn, these results should help decrease the chronic under-diagnosis of rickettsial diseases throughout the Americas.

This work was supported by work unit no. 800000.82000.25GB. B0016, Promis ID#MLEguia-PO166-14, for FY2014, from the Global Emerging Infections Surveillance and Response System of the Armed Forces Health Surveillance Center.

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**Table. Multilocus sequence typing analysis of complete genes from Peru Rickettsia asembonensis molecular isolate VGD7**

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<th>Changes</th>
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<th>Variable genes (GenBank accession no.)</th>
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<td>ompA (KY650698) ompB (KY650699) sca4 (KY650700)</td>
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<tr>
<td></td>
<td>Identity, %</td>
<td>Identity, %</td>
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<td>Mutations</td>
<td>Total changes</td>
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<tr>
<td></td>
<td>Identity, %</td>
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</tr>
<tr>
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<td>Mutations</td>
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<tr>
<td>Protein</td>
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<tr>
<td></td>
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<td>Identity, %</td>
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<tr>
<td></td>
<td>Mutations</td>
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</tr>
</tbody>
</table>

*Comparisons were made against reference strain NMRJ3a from Kenya (GenBank accession no. JWSW01000078.1). Blank cells indicate no additional mutations or changes to report. ORF, open reading frame.*
About the Author

Mr. Loyola is a researcher in the Genomics & Pathogen Discovery Unit at the US Naval Medical Research Unit No. 6, in Lima, Peru. His primary research interest is the characterization of new and emerging pathogens.

References


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negative by real time RT-PCR (details on laboratory testing in online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/17-1479-Techapp1.pdf). We detected Zika virus IgM and IgG by a commercial immunofluorescence assay (see online Technical Appendix).

The patient was offered a chorionic villi sampling; the genetic analysis was normal. Surgical evacuation of the uterus was performed by vacuum aspiration followed by curetage. We detected Zika virus by real time RT-PCR in both the transport medium in which the chorionic biopsy was stored ($C_t = 36$) and the supernatant of the karyotype cell culture ($C_t = 12$). Differences in real-time PCR $C_t$ values can be explained by active viral replication in the karyotype cell culture. We used the supernatant of the karyotype cell culture to inoculate Vero cells, where we observed a cytopathic effect. We confirmed virus isolation by subsequent infection of new Vero cells, RT-PCR analysis, and sequencing of the Zika virus envelope gene. This analysis suggested active Zika virus replication in embryonic cells. We also detected Zika virus by real time RT-PCR in fresh placental tissue samples from vacuum aspiration (online Technical Appendix).

Formalin-fixed paraffin-embedded placental tissues were also analyzed at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). Histopathological analyses of these placental tissues revealed perivillous fibrinoid deposition, focal coarse calcifications, and moderate increase of Hofbauer cells. The histological sections of the placental tissue, which were stained with hematoxylin and eosin, showed a focus of villous necrosis associated with calcifications. A small portion of embryonic membranes was visible, showing no noteworthy inflammatory infiltrate. Immunohistochemical testing on placental tissue did not show presence of Zika virus–specific immunostaining. The histological findings were not relevant to the diagnosis. No specific changes were observed, neither associated inflammation was identified, and only nonspecific mild abnormalities were present. Nevertheless, Zika virus RT-PCR assays and sequencing performed on RNA extracted from placental tissues identified the presence of Zika virus in the sample ($C_t = 37$). On July 6, 21 days after vacuum aspiration and 31 days after the onset of symptoms, we detected Zika virus in maternal serum samples using RT-PCR ($C_t = 37$).

Our investigation found evidence of Zika virus infection in tissue samples from an early pregnancy loss in a mother infected with Zika virus in the first trimester of pregnancy.

**Figure.** Clinical timeline for a 22-year-old pregnant woman who had suspected Zika virus infection. The woman was in the seventh week of gestation when she traveled from Spain to the Dominican Republic. CRL, crown–rump length; DENV, dengue virus; Ig, immunoglobulin; IHC, immunohistochemistry; ZIKV, Zika virus; RT-PCR, reverse transcription PCR; +, positive; −, negative.
Testing of tissues from vacuum aspiration and from chorionic villi sampling revealed that placenta and chorion contained Zika virus RNA. Isolation of Zika virus from the karyotype cell culture confirmed active viral replication in embryonic cells. All the tests performed suggest that the spontaneous abortion in this woman was likely associated with a symptomatic Zika virus infection occurring early in pregnancy. These findings provide further evidence of the association between Zika virus infection early in pregnancy and transplacental infection, as well as embryonic damage, leading to poor pregnancy outcomes (2). Given that embryo loss had probably occurred days before maternal-related symptoms, we hypothesize that spontaneous abortion happened early during maternal viremia. The prolonged viremia in the mother beyond the first week after symptom onset concurs with other recent reports (1,5). However, persistent viremia 3 weeks after pregnancy outcome has not been described previously and underscores the current lack of knowledge regarding the persistence of Zika virus infection. Because we identified Zika virus RNA in placental tissues, our findings reinforce the evidence for early gestational placental tissue as the preferred target for viral tropism (2,4). Finally, although laboratory tests were performed to dismiss other maternal infections (see online Technical Appendix), the attribution of Zika virus as the cause of the spontaneous abortion must be interpreted with caution, because a non–Zika-related etiology cannot be entirely ruled out. Further studies are warranted to investigate the natural history of Zika virus infection in pregnant women.

This work was funded by grant no. PI16/0123. ISCIII-AES-Proyectos de Investigación en Salud, 2016, Government of Spain.

About the Author
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References

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Isolation of Oropouche Virus from Febrile Patient, Ecuador

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DOI: https://doi.org/10.3201/eid2405.171569

We report identification of an Oropouche virus strain in a febrile patient from Ecuador by using metagenomic sequencing and real-time reverse transcription PCR. Virus was isolated from patient serum by using Vero cells. Phylogenetic analysis of the whole-genome sequence showed the virus to be similar to a strain from Peru.

Oropouche virus (OROV) is a negative-sense, single-stranded RNA virus (family Bunyaviridae, genus Oropouchevirus) with a tripartite genome consisting of large (L), medium (M), and small (S) segments. OROV causes a self-limiting acute febrile illness, Oropouche fever (1). Since its discovery in Trinidad in 1955 (2), >30 outbreaks of OROV have been reported from Brazil, Panama, and Peru, demonstrating the ability of this midgeborne virus to cause epidemics. Approximately 500,000 cases of Oropouche fever have been reported, making OROV one of the most clinically significant orthobunyaviruses (1). Two previous studies reported unconfirmed infections in Ecuador by using...
serologic or antigenic evidence (3,4). We describe whole-genome sequencing and virus isolation of OROV in Ecuador.

We collected a blood sample from a consenting 41-year-old male patient in Esmeraldas, Ecuador, who sought treatment in April 2016 after 7 days of fever, headache, joint pain, muscle pain, and nausea. The patient reported that he had been in Esmeraldas for ≥3 months and had not traveled outside the province during that time. RNA was extracted from plasma of the blood sample and tested at Universidad San Francisco de Quito, Ecuador, and Public Health England, UK, for dengue virus (DENV), chikungunya virus (CHIKV), Zika virus, yellow fever virus, Mayaro virus, Plasmodium spp., Leptospira spp., and Rickettsia spp. by using real-time reverse transcription PCR (rRT-PCR) and conventional RT-PCR assays developed in-house or acquired commercially (Genesig, Primerdesign Ltd., Cambridge, UK). The sample gave borderline results for DENV (quantitation cycle [Cq] 35.3) and CHIKV (Cq 36.6; reference ranges ≤35 positive, 35–40 borderline, >40 negative) and negative results for the other pathogens.

As an initial screen for other pathogens, we applied unbiased metagenomic sequencing. Analysis of sequencing reads by using Kraken, a system for assigning taxonomic labels to individual reads (5), identified 1% reads (5,016 of 464,444) as specific to OROV. We generated an OROV consensus sequence by mapping reads to a reference sequence, which resulted in coverage of 69% for S, 76% for M, and 79% for L OROV viral RNA segments (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/24/2/17-1569-Techapp1.pdf). We classified 1,228 reads as DENV serotype 1, all of which mapped to a single 732-nt region of the DENV-1 reference genome. No reads mapped to CHIKV.

After confirmation of the presence of OROV by using a validated rRT-PCR (6), we attempted to isolate OROV by using Vero and C6/36 cell lines inoculated with the patient’s plasma. We confirmed virus replication by detecting increasing OROV RNA over time by using rRT-PCR. We obtained whole-genome sequences by sequencing viral RNA from harvested OROV supernatant; each genome segment was sequenced at average depths of coverage of 55,532× for S, 4,954× for M, and 5,674× for L segments. We deposited sequences in GenBank (online Technical Appendix). Genetic organization was similar to that of other OROV strains: segment lengths 952 nt for S, 4,387 nt for M, and 6,852 nt for L.

Phylogenetic analysis (online Technical Appendix) showed that the virus we isolated, OROV/EC/Esmeraldas/087/2016, was most closely related to a strain isolated from a patient in Peru during 2008 and excluded the possibility of the virus being a reassortant orthobunyavirus, such as Iquitos virus. This finding suggests a potential introduction across the Peru–Ecuador border; however, further investigation is required to understand the origin and incidence of OROV in Ecuador. The known urban OROV vector, the Culicoides paraensis midge, is absent in the Pacific Coast region, including Esmeraldas (S. Zapata, pers. comm., 2017 Aug 31), which raises the question of alternative insect vectors in OROV transmission. Culex mosquitoes have previously been implicated as vectors in the OROV urban cycle, notably C. quinquefasciatus (1), a species that is widespread throughout South America (7).

DENV and CHIKV rRT-PCR results for this patient were inconclusive. The small proportion of DENV reads in the metagenomic data suggests DENV-1 infection is possible. Using ELISA to detect DENV and CHIKV-specific antibodies may help clarify the results.

It is likely that cases of Oropouche fever go unreported or misdiagnosed. Clinical features of the disease are similar to those of other viral, protozoan, and bacterial diseases previously reported in Ecuador (1,4,8,9). OROV might spread unnoticed across a wide geographic area, as suggested by this unexpected detection. Several studies have successfully documented the use of metagenomic sequencing for virus identification in febrile patients (10); this approach is becoming more practicable as costs decrease, the major benefit being the ability to detect unexpected or novel viral sequences, as evidenced by this detection of OROV.

This work highlights the need for increased surveillance of OROV in Ecuador and effective differential diagnostic assays to distinguish between emerging pathogens sharing common clinical descriptions to those already circulating. To clarify the true prevalence of this disease in Ecuador, the OROV rRT-PCR assay will be used to screen archived and newly collected samples from a cohort of patients seeking treatment for acute undifferentiated febrile illness during 2016–2017.

Acknowledgments

The authors thank Liana Kafetzopoulou and Kuiama Lewandowski for providing technical support.

This study was approved by the bioethics committee of Universidad San Francisco de Quito. The patients provided written consent indicating that they agreed for their samples to be tested for additional pathogens.

Ms. Wise is a PhD student registered with Plymouth University and funded by Public Health England. Her research interests are emerging viral infections and viral immunology.

References


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DOI: https://doi.org/10.3201/eid2405.ET2405

**Oropouche [o’ro-poo”che] Virus**

Ronnie Henry and Frederick A. Murphy

In September 1955, a virus was isolated from a 24-year-old forest worker from the community of Vega de Oropouche, near the town of Sangre Grande, on the island of Trinidad (country: Trinidad and Tobago), who presented with fever, backache, and cough, which resolved spontaneously after 3 days. The virus was isolated from the patient’s blood by intracranial inoculation at the Trinidad Regional Virus Laboratory. Five years later, the virus was isolated from a 24-year-old forest worker from the community of Vega de Oropouche, near the town of Sangre Grande, on the island of Trinidad (country: Trinidad and Tobago), who presented with fever, backache, and cough, which resolved spontaneously after 3 days. The virus was isolated from the patient’s blood by intracranial inoculation at the Trinidad Regional Virus Laboratory.

The virus was shown to be unique but antigenically related to Simbu virus, which had recently been described from South Africa. It therefore became a member of the large family of bunyaviruses.1

The virus, Oropouche virus, named in keeping with the tradition of designating arboviruses by using local geographic names, stems from the name of the village, a nearby swamp (wetland), and river. It derives from an Amerindian word, but the ancient meaning of the word is not clear.

Oropouche virus has since proven to be one of the most common arthropodborne viruses infecting humans in the tropics of the Western Hemisphere. Clinical signs of infection include headache, myalgia, arthralgia, and chills; no deaths have been reported. It has been estimated to have infected more than half a million persons in Brazil alone, and there have also been large outbreaks in Panama and Peru. In keeping with the recent emergence of other arboviruses such as West Nile, chikungunya, and Zika viruses, Oropouche virus is a candidate for possible further urban spread and therefore warrants increased surveillance and diagnostics.

In 2017, the International Committee on Taxonomy of Viruses (ICTV) reclassified the family Bunyaviridae, elevating the family to an order. The formal taxonomic placement of Oropouche virus is order Bunyavirales; family Peribunyaviridae; genus Orthobunyavirus; species: Oropouche orthobunyavirus.

### Sources


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A Mental Models Approach to Assessing Public Understanding of Zika Virus, Guatemala

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Mental models are cognitive representations of phenomena that can constrain efforts to reduce infectious disease. In a study of Zika virus awareness in Guatemala, many participants referred to experiences with other mosquito-borne diseases during discussions of Zika virus. These results highlight the importance of past experiences for Zika virus understanding.

Current risk communication literature includes guidelines regarding crises (1,2). Creating and distributing risk-mitigation information amid a nexus of emotion, public health threats, a journalistic tendency toward sensationalism, and misinformation can be daunting (3,4), which can make general guidelines appealing. We argue here, though, that tension between widely held, preexisting mental models of disease and the circumstances of each new emergent infectious disease offers an underappreciated challenge. By better acknowledging how existing audience mindsets can reflect past experiences sometimes at odds with new circumstances, we can move beyond set guidelines to call for formative research, psychologically oriented literature reviews, and social discourse monitoring as crucial steps to address emerging infectious diseases. To support our argument, we offer case evidence regarding public understanding of Zika virus in Guatemala in early 2016.

Any assessment of public health intervention potential can begin with understanding existing mental models among a population. Mental models involve how persons imagine and conceptualize phenomena (5,6; online Technical Appendix, https://wwwnc.cdc.gov/EID/article/24/5/17-1570-Techapp1.pdf). In the case of infectious disease, mental models can include conceptualization of disease transmission routes and processes and constraints, just as mental models of mechanical tools can include a person’s understanding of engineering principles.

To clarify how Guatemalans perceived Zika virus in spring 2016, we conducted 8 focus groups (with separate groups for men and women) and 10 in-depth individual interviews (coordinated in country by T.L.). Participants were adults 18–49 years of age in both urban and rural regions affected by the virus. We recruited participants from the database of a market research firm in Guatemala (ConsuMer). Staff conducted all focus groups and interviews in Spanish in 2 departments (Zacapa and Suchitepéquez) (7); focus groups took place in central locations (e.g., restaurants) and individual interviews occurred in participants’ homes.

The importance of personal experience with mosquito-borne viral disease in informing Zika virus understanding was striking. Virtually all participants were aware of Zika as a disease affecting Guatemala at the time of the interviews. At the same time, much of the discussion with participants clearly referred to other mosquito-borne diseases, rather than their conceiving of Zika virus as a new pathogen.

This pattern is understandable given the disease context in Guatemala, where dengue fever has been endemic for years, and given the substantial outbreak of chikungunya disease in 2014. Zika virus emerged and began to spread rapidly in Guatemala in late 2015. Participants apparently drew on experience with dengue and chikungunya as a baseline in understanding Zika virus. Mosquitoes were most commonly mentioned as a vector for the Zika virus, and participants often pointed out that the type of mosquito that transmitted Zika also was responsible for chikungunya and dengue.

Some participants made distinctions between mosquito-borne diseases. Many participants (≥1 in each of all 8 focus groups and 6 in individual interviews), for example, noted that the presence of conjunctivitis (red eyes) distinguishes Zika from other mosquito-borne diseases. Even in such cases, however, dengue and chikungunya served as an anchoring reference against which Zika virus was compared. Anchoring bias is a human tendency to rely on an initial piece of information even when new information comes to light (8).

Such predominant understanding of mosquito-borne transmission appears to have overshadowed other possibilities for transmission routes in popular imagination. Fewer than half of the in-depth interview participants reported knowing that Zika virus could be sexually transmitted. That gap in understanding poses challenges to preventive efforts to change social interaction (as opposed to emphasizing prevention of interaction with mosquitoes). Moreover, many participants (≥1 in each of 6 focus groups and half in individual interviews) expressed some sense of inevitability regarding mosquito-borne disease, likely because avoiding mosquito bites in Guatemala at certain times of the year can be difficult.

Anecdotal experience with symptoms loomed large in discussion. Participants (≥1 in each of 4 focus groups) noted, after learning about sexual transmission possibilities, that they would look for symptoms in a partner and base sexual behavior on their assessment (insofar as they had agency to...
decide). We know, however, that a person infected with Zika virus might not have easily observable symptoms. Even if persons accept the possibility of sexual transmission, they might not engage in safe sex practices with asymptomatic infected partners.

If persons understand Zika virus through a mental model informed by dengue or chikungunya, public health officials should address potential confusion, especially in light of differences (e.g., sexual transmission) that might be misunderstood or ignored. Even when not confusing the illnesses, participants clearly conceptualized Zika in comparison with relatively more familiar illnesses. In this way, they operated in similar fashion as consumers encountering novel products do (9,10). Public health messaging might leverage this tendency. If it is easiest to understand a new outbreak in comparison to a previous one, using analogy or direct comparison might be effective but will also require careful emphasis on what is new.

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Cerebrospinal Fluid Immunoglobulins as Potential Biomarkers of Chikungunya Encephalitis

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Chikungunya virus causes fever and severe polyarthritis or arthralgia and is associated with neurologic manifestations that are sometimes challenging to diagnose. We demonstrate intrathecal synthesis of chikungunya antibodies in a patient with a history of acute infection complicated by encephalitis. The specificity of the intracerebral immune response supports early chikungunya-associated encephalitis diagnosis.

Chikungunya virus (CHIKV) is an alphavirus transmitted by infected Aedes mosquitoes (Ae. aegypti and Ae. albopictus) (1). Global expansion epidemics have been
reported (1). The disease is characterized by acute fever, maculopapular rash, headache, and disabling rheumatism (1,2). Neurologic complications may occur, including encephalopathy, encephalitis, myelitis, and Guillain-Barré syndrome (3–5). Differentiating CHIKV infection from other arbovirus infections is difficult because of co-occurring conditions and similar manifestations (3). Detection of viral RNA and specific antibodies in cerebrospinal fluid (CSF) suggests neurotropic evidence of CHIKV (2,3), but whether these antibodies are synthesized locally or derived from blood has not been demonstrated (6). We detected intrathecal synthesis of CHIKV IgG by specific antibody index in a case of encephalitis (6).

In January 2016, a 69-year-old woman had sudden fever (38°C), intense generalized arthritis, prostration, and cognitive alterations characterized by forgetting and exchanging words. She used analgesics without relief. After 1 week, a maculopapular rash with intense pruritus appeared on her upper limbs. A few days later, her rash and fever abated, but other symptoms continued. She was referred for consultation 3 months after symptom onset. Physical examination revealed bilateral finger and knee arthritis (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/24/5/17-1763-Techapp1.pdf). Neurologic examination showed slow thinking, inattention, and mild confusion. She had a history of dengue and Zika virus infections. Results of a routine blood analysis were unremarkable.

We performed several antibody tests: anti-CHIKV ELISA (IgM and IgG; EUROIMMUN, Luebeck, Germany); Panbio Dengue IgG Indirect ELISA and Dengue IgM Capture ELISA (Panbio, Brisbane, Queensland, Australia); and immunochromatographic assay (GenBody Zika IgG/IgM; Biotech Business, Chungham, South Korea). Results for specific IgG and IgM are shown in the Table. CSF analysis demonstrated leukocytes 1 cell/mm³ protein 29 mg/dL; glucose 40 mg/dL; blood–CSF barrier function based on albumin quotient 6 × 10⁻³; IgG index 0.43 (reference <0.7); and intrathecal IgG fraction (IgGᵢₜ) of total IgG found in CSF, <0% (6,7). In addition, we quantitatively determined the synthesis of specific antibodies by antibody index (AI) (6). We used an ELISA test for DENV IgG and ELISA for CHIKV IgG in paired CSF and serum. We calculated AI as the ratio between the specific IgG and total IgG quotient, considering that there was no intrathecal synthesis of total IgG (IgGᵢₜ <0%). The sample dilutions for dengue were 1:8 for CSF and 1:4,000 for serum. Sample dilutions for CHIKV were 1:2 for CSF and 1:101 for serum (6,8). AI was 1.14 for dengue and 7.24 for CHIKV, with AI reference range <1.5 (online Technical Appendix).

Brain magnetic resonance imaging showed foci with hyperintense signal in the T2-weighted sequences and fluid attenuation inversion recovery bilaterally in subcortical frontoparietal areas. The patient showed substantial progressive improvement of cognitive alterations and arthralgia after starting antiinflammatory treatment; she took nimesulide for 2 months, followed by prednisolone (20 mg/d) with progressive reduction for another 2 months.

Our results demonstrate the quantitation of intrathecally synthesized CHIKV IgG. We diagnosed CHIKV-associated encephalitis on the basis of fever and altered mental status for >24 hours and positive CHIKV IgM antibodies in serum (9). CSF analysis results were unremarkable except for elevated CHIKV AI, the only evidence of brain inflammation. Brain imaging showed unspecific lesions; viral encephalitis may occur without pleocytosis or specific parenchymal abnormalities (9).

The identification of specific etiologies of viral encephalitis may be difficult in arbovirus-endemic areas (3). Co-infections with Zika virus, CHIKV, and DENV are frequent (1,3), and neurologic manifestations may also be similar (4,5). Although this case-patient also had a history of DENV and Zika infection with specific IgG in serum and CSF, we did not detect intrathecal synthesis of DENV antibodies (6–8) as did Puccioni-Sohler et al. in a previous study (8). Our findings show that the quantitation of antibodies synthesized in the brain may be useful in the differential diagnosis of neurologic diseases caused by arboviruses. The detection of specific intrathecal synthesis of antibodies is a known tool for the diagnosis of infections including herpes simplex virus, varicella zoster virus, measles, rubella, neuroborreliosis, and human T-cell leukemia virus type 1 (6–8).

CHIKV has attracted increasing attention because of its spatial spread and the high number of epidemics. Chikungunya has been associated with debilitating arthropathy for months or years after the initial infection, along with severe neurologic complications such as encephalitis (4,5,10). The detection of the etiologic agent of a central nervous system disease may be difficult, considering that PCR results for CHIKV are positive only during the first days of infection (1). In addition, the presence of specific antibodies in CSF may be derived from blood (6,7). The detection of intrathecal synthesis of CHIKV IgG may be useful as a specific laboratory brain marker for diagnosis of encephalitis and other neurologic complications associated with CHIKV infection. This result provides evidence of viral neurotropism and can be useful for supporting public health.

Table. Results of the detection of IgM and IgG against chikungunya virus, dengue virus, and Zika virus, Brazil, 2016

<table>
<thead>
<tr>
<th>Target virus</th>
<th>Serum</th>
<th>Cerebrospinal fluid</th>
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<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
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<tr>
<td>Chikungunya</td>
<td>Reactive</td>
<td>Not reactive</td>
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<tr>
<td>Dengue</td>
<td>Reactive</td>
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<tr>
<td>Zika</td>
<td>Not reactive</td>
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Author contributions: M.P.-S. drafted the study concept and acquired and interpreted data; L.C.F., M.J.C.-C., and M.C.F.S. acquired and conducted laboratory analyses; M.P.-S., M.G.Z., and M.C.F.S. revised the manuscript; and R.S.K. conducted neuropsychological tests included as supplementary data.

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Chronic Genotype 3 Hepatitis E in Pregnant Woman Receiving Infliximab and Azathioprine
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Acute hepatitis E virus infection during pregnancy has a high fatality rate in developing countries. Little data are available on chronic infection in pregnant women. We report a case of chronic hepatitis E during treatment with infliximab and azathioprine, without adverse event during pregnancy and with spontaneous resolution after delivery.

Hepatitis E virus (HEV) genotype 1 causes a high number of deaths of pregnant women in developing countries (1). The few reported cases of HEV during pregnancy in industrialized countries (2–5) mainly relate to acute genotype 3 infection. We report the course of autochthonous
chronic genotype 3c (GenBank accession no. KX602217) hepatitis E in a pregnant woman in France.

The patient, 27 years of age, was receiving immunosuppressive therapies for ulcerative colitis and became pregnant during the infection and treatment. At symptom onset, she had received infliximab and azathioprine for >5 years and reported eating undercooked meat; she had not traveled abroad. Prolonged elevated alanine aminotransferase (ALT) since May 2014 led her physician to suspect viral hepatitis; HEV infection was later diagnosed in September 2014 by detection of HEV IgM and RNA in plasma (Figure, panel A). We retrospectively tested previous blood samples from this patient, routinely stored in the hospital virology laboratory, and found them to be negative for HEV IgM and RNA.

Persistence of HEV has not been reported among patients receiving infliximab or azathioprine. However, HEV persistence was reported in a patient receiving azathioprine combined with oral steroids (6) and in a pig model of HEV chronicity under combined cyclosporine/azathioprine/methylprednisolone (7). On the basis of those reports, we reduced the patient’s dose of azathioprine to 100 mg/d and that of infliximab to 5 mg/kg/d every 8 weeks in November 2014 (Figure, panel B), but infection did not resolve. She became pregnant shortly thereafter.

During the patient’s pregnancy, viral loads ranged from 5.7 to 6.8 log_{10} copies/mL, and ALT returned to reference range (Figure, panel A). We discontinued infliximab at the beginning of the third trimester (Figure, panel B). Viral load increased by >1 log_{10} copies/mL, and ALT remained within reference range. She gave birth by vaginal delivery at 40 weeks of amenorrhea. On the day of delivery, 3 months after infliximab discontinuation, viral load peaked (6.9 log_{10} copies/mL; Figure, panel A). Although viral load was high during pregnancy, the infant was not infected and was in good health; HEV RNA was undetectable in cord blood (the placenta was not available for evaluation), and neither HEV IgM nor RNA were found in the newborn’s plasma 2 days after birth.

After delivery, testing of the mother’s plasma showed cytolysis (ALT >3× upper limit of reference range) and a >3-log decrease of HEV RNA (Figure, panel B). We reintroduced infliximab 3 weeks after delivery, at which time HEV RNA was lower than during pregnancy but still detectable (3.5 log_{10} copies/mL; Figure, panel A). At 2 months after delivery, hepatic cytolysis resolved; 2 months later, HEV became undetectable. No relapse was noted during subsequent follow-up (the last PCR performed in August 2017 was negative).

Because of the high rate of severe acute hepatitis E reported in pregnant women in developing countries, we monitored the patient for negative outcomes during gestation but found none. This finding is consistent with the small number of reported HEV infections during pregnancy in industrialized countries (2–5), despite high seroprevalence in the general population (8,9).

Innate immunity has been suggested as essential for severe outcomes of acute HEV infection during pregnancy (10). In our study, HEV infection had moved toward chronic infection before pregnancy, which may have reduced the role of innate immunity. T-cell responses are decreased in immunosuppressed patients and in pregnant women, Figure. Time courses of HEV viral load (A) and infliximab and azathioprine treatment (B) in pregnant woman with chronic hepatitis E who was undergoing immunosuppressive treatment for ulcerative colitis. HEV RNA levels, IgM and IgG positivity, and serum ALT levels are shown. Serum ALT is expressed as a multiple of ULN. Arrows indicate suspected and confirmed infection and delivery dates. Infliximab treatment occurred every 8 weeks. ALT, alanine aminotransferase; HEV, hepatitis E virus; ULN, upper limit of normal.
particularly when term approaches. The imbalance in T-cell immunity (Th1/Th2) has been proposed to be implicated in the progression of chronic HEV infection in immunocompromised pigs (7). This imbalance may explain the absence of cytolysis during pregnancy and the increased viral load observed despite discontinuation of infliximab. Conversely, after delivery, restoration of cellular immunity is commonly observed (11) and may have contributed to efficient clearance of the virus by hepatic cytolysis along with the reduced immunosuppression resulting from infliximab discontinuation. Despite re-introduction of infliximab when HEV RNA was still detectable, we observed spontaneous resolution of chronic hepatitis E, although immunosuppressive treatment at that time was identical to that previously implicated in the chronicity of infection.

The risk for HEV vertical transmission seems dependent on viral load (12). In a model of HEV infection in pregnant rabbits, Xia et al. reported severe outcomes and a high level of transmission to offspring (13). In the case we report, despite high viral loads in the mother’s plasma throughout pregnancy, we found no HEV RNA in the newborn’s plasma. Of note, although mothers in the rabbit model were negative for HEV IgG throughout pregnancy, in the case we report, the mother was IgG positive before pregnancy, which may have helped protect the fetus from infection, although this protective role is inconsistent in previous reports of HEV genotype 3 (HEV3) infection of humans (2–4). Furthermore, despite a high sequence similarity to HEV3, rabbit HEV cross-species infections are restricted to nonhuman primates, and pathogenesis may differ from that of HEV3. In conclusion, our results and those reported by Mallet et al. (5) indicate that chronic HEV3 infection in pregnant women might resolve after pregnancy.

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Multiple Introductions of Influenza A(H5N8) Virus into Poultry, Egypt, 2017


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After high mortality rates among commercial poultry were reported in Egypt in 2017, we genetically characterized 4 distinct influenza A(H5N8) viruses isolated from poultry. Full-genome analysis indicated separate introductions of H5N8 clade 2.3.4.4 reassortants from Europe and Asia into Egypt, which poses a serious threat for poultry and humans.

In Egypt, highly pathogenic avian influenza A(H5N1) clade 2.2.1 virus was introduced to poultry via migratory birds in late 2005 (1) and is now endemic among poultry in Egypt (2). Also in Egypt, the number of H5N1 infections in humans is the highest in the world, and low pathogenicity influenza A(H9N2) virus is widespread among poultry and has infected humans (2). Despite extensive vaccination, H5N1 and H9N2 viruses are co-circulating and frequently reported (2). In 2014, highly pathogenic avian influenza A(H5N8) virus clade 2.3.4.4 was isolated, mostly from wild birds, in several Eurasian countries and was transmitted to North America. However, in 2016 and 2017, an unprecedented epidemic was reported in Asia, Africa, and Europe (3). In Egypt, during November 30–December 8, 2016, a total of 3 H5N8 viruses were isolated from common coot (Fulica atra) (4) and green-winged teal (Anas carolinensis) (5). To provide data on the spread of the virus in poultry, we genetically characterized 4 distinct H5N8 viruses isolated from commercial poultry in Egypt in 2017.

During February–May 2017, a high mortality rate was observed for 48 poultry flocks in the Nile Delta, Egypt. Up to 20 tracheal and cloacal swab samples were collected from each flock for initial diagnosis by reverse transcription PCR and virus isolation at the Faculty of Veterinary Medicine, Damanhour University (Damanhour, Egypt). Results were positive for H5N8 virus in samples for 4 flocks not vaccinated for H5 in 3 governorates (Figure). Sudden deaths also occurred in 3 broiler chicken flocks (Ck12, Ck15, Ck21) and 1 duck flock (Dk18); mortality rates were 29%–52% (online Technical Appendix 1 Table 1, https://wwwnc.cdc.gov/EID/article/24/5/17-1935-Techapp1.pdf). No epidemiologic links between farms were observed.

Positive samples were spotted onto FTA cards (6) and submitted to Friedrich-Loeffler-Institut (Insel Riems-Greifswald, Germany), where H5N8 virus was confirmed...
by reverse transcription PCR and full-genome sequences (7) from 4 viruses (GISAID [https://www.gisaid.org/] accession nos. EP11104268–EP11104299) (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/24/5/17-1935-Techapp2.pdf). We retrieved sequences with high similarity and all H5N8 virus sequences from GISAID and GenBank and aligned them by Multiple Alignment using Fast Fourier Transform (https://mafft.cbrc.jp/alignment/server/index.html). The most highly related viruses are summarized in online Technical Appendix 1 Table 2. We calculated sequence identity matrices in Geneious (https://www.geneious.com/) (online Technical Appendix 1 Figure 1) and studied phylogenetic relatedness to H5N8 virus isolated in Eurasia and in Egypt by using IQtree (http://www.iqtree.org/). Representative viruses were selected for generation of maximum-likelihood midpoint rooted trees by MrBayes (http://mrbayes.sourceforge.net/) using a best-fit model (GTR+G) (8) and were further edited by using FigTree (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape (https://inkscape.org/en/).

The hemagglutinin (HA) and neuraminidase (NA) genes of the 4 viruses shared 95.8%–99.2% nt and 93.1%–99.4% aa identity and shared 96.5%–99.2% nt and 94.2%–99.7% aa identity with viruses from wild birds in Egypt (4,5). Other segments showed 92.6%–99.6% nt and 96%–99.7% aa identity, where the polymerase acidic (PA) genes and proteins of viruses from Dk18 showed the lowest similarity to those of other viruses (online Technical Appendix 1 Figure 1).

HA of the 4 H5N8 viruses in this study clustered in 1 distinct branch (Figure), and NA clustered in 2 phylogroups (online Technical Appendix 1 Figure 2). The PB2, nucleoprotein, M, and NS genes of viruses from Ck12 and Ck15 (from chickens in the same governorate, February and May 2017) clustered together, and the same genes from viruses from Dk18 and Ck15 (from ducks and chickens in 2 governorates) clustered in 2 distinct phylogenetic groups. However, viruses from Ck12 and Ck15 have similar but not identical PA gene segments (online Technical Appendix 1 Figure 3).

These data suggest 4 different introductions of H5N8 virus into poultry in Egypt, independent of viruses isolated from captive birds (4,5). Multiple separate introductions of H5N8 virus into Europe also occurred (10). Further studies are needed to identify the source(s) of introduction. The separate introductions of different reassortants of H5N8 clade 2.3.4.4 virus from Europe and Asia into Egypt indicate a serious threat for poultry and human health.

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Fatal Tick-Borne Encephalitis Virus Infections Caused by Siberian and European Subtypes, Finland, 2015

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In most locations except for Russia, tick-borne encephalitis is mainly caused by the European virus subtype. In 2015, fatal infections caused by European and Siberian tick-borne encephalitis virus subtypes in the same *Ixodes ricinus* tick focus in Finland raised concern over further spread of the Siberian subtype among widespread tick species.

The causative agent of tick-borne encephalitis (TBE), *tick-borne encephalitis virus* (TBEV), is endemic throughout Europe and Asia; >10,000 cases are reported annually (1). TBEV is an enveloped, positive-sense RNA virus in the family *Flaviviridae*, genus *Flavirus* (2). The westermmost range of the Siberian subtype (TBEV-Sib) extends to Finland and the Baltics, where the European subtype (TBEV-Eur) also circulates. TBEV-Eur is the only subtype found in the rest of Europe (3).

In TBEV-infected patients, neurologic signs appear as the virus passes to the central nervous system; infection is manifested as meningitis, encephalitis, or meningoencephalitis. During 2010–2016, a total of 20 cases of TBE were reported from Kotka archipelago, Finland, a previous TBEV-Sib focus (4). We report 2 fatal TBEV infections acquired 1 month apart in patients on Kuutsalo Island, Kotka archipelago, in 2015.

Patient 1 was a previously healthy 36-year-old woman who had visited Kuutsalo 10 days before fever onset. A week later, she experienced sudden-onset headache, left arm numbness, and impaired vision. Head computed tomography results were unremarkable. Two days later, she experienced disorientation and right hemiparesis and was taken to a tertiary care center. Cerebrospinal fluid (CSF) test results showed pleocytosis. Magnetic resonance images indicated pathologically increased signal in cortical sulcus regions (Figure, panel A). Despite receipt of acyclovir, doxycycline, and ceftriaxone, her condition deteriorated rapidly. Head computed tomography showed cerebellar herniation; the patient had dilated pupils and no pain reaction. CSF and serum were positive for TBEV IgM but negative for TBEV RNA; hemagglutination inhibition results showed a low titer (20) of TBEV-specific antibodies in serum. The patient died 2 weeks after fever onset.

Gross postmortem examination showed widespread and severe signs of viral encephalitis: meningeal and perivascular inflammation, neuronophagy, microglial nodules, endothelial damage, and severe brain edema. The inflammation was evident from the spinal cord to the cerebellum and cortex (Figure, panel B). TBEV (RNA) was detected in brain and spleen (online Technical Appendix Figure 1, panel A, https://wwwnc.cdc.gov/EID/article/24/5/17-1986-Techapp1.pdf).

TBEV was isolated from the cerebellum in SK-N-SH neuroblastoma cells, and the whole genome for TBEV-Sib was obtained. A pool of TBEV-Sib–positive *Ixodes ricinus* ticks collected from the neighboring island in 2011 (4) was subjected to viral whole-genome sequencing. This virus and the virus from patient 1 had 3 nt differences resulting in 2 aa mutations, R868K (NS1) and V1452A (NS2B), and clustered together in the Baltic clade of TBEV-Sib (online Technical Appendix Figure 2).

Patient 2 was a 66-year-old man with hypertension, diabetes, and chronic lymphatic leukemia. He had frequently...
been bitten by ticks while at his cottage on Kuutsalo Island. Two weeks before hospitalization, he had persistent fever. By the time he was hospitalized, tetraparesis and urinary retention had developed. Magnetic resonance images showed increased signal in cerebellar vermis, facial nerves, cortical sulci, and radicular regions (Figure, panel C). CSF analysis showed pleocytosis. Serum and CSF were negative for TBEV IgM and RNA. The patient’s condition deteriorated rapidly; tetraplegia developed, and he lost consciousness despite treatment with acyclovir, doxycycline, ceftriaxone, plasmapheresis, and immunoglobulin. One week after hospitalization, his CSF was positive for TBEV IgM but his CSF, serum, and urine were RNA negative. Hypogammaglobulinemia was observed. The patient died 4 weeks after hospitalization.

Postmortem examination showed signs of severe coronary disease, cardiac hypertrophy, atherosclerosis in the aorta, and bronchopneumonia. Examination for neuropathology showed abundant perivascular lymphocytosis continuing to brain parenchyma causing glial reactivity and neuronophagy, altogether demonstrating viral encephalitis prominent in the spinal cord, brain stem, basal ganglia, and cerebellum (Figure, panel D). The brain was positive for TBEV RNA (online Technical Appendix Figure 1, panel B). A complete genome for TBEV-Eur was sequenced from the cerebellum (online Technical Appendix Figure 2).

In September 2017, a total of 80 ticks were collected from Kuutsalo Island. One, collected at the cottage of patient 2, was positive for TBEV RNA. The virus was isolated in SK-N-SH cells, and a TBEV-Eur genome was sequenced. This virus and the virus from patient 2 had 6 nt differences, resulting in 1 aa difference (F2995Y).

For both patients, progression of TBE was rapid and aggressive; neither patient had been vaccinated. Patient 1, who was young and previously healthy and who was infected with TBEV-Sib, died of brain herniation. Patient 2, who had predisposing conditions, was infected with TBEV-Eur and died of tetraplegia and subsequent complications.

In Finland, TBEV-Eur has been found atypically in I. persulcatus ticks, and TBEV-Sib has been found in I. ricinus ticks (4,5). TBEV-infected I. ricinus ticks are typically found in Kotka archipelago. The detection of TBEV-Eur from patient 2 was unexpected in a known TBEV-Sib focus. The high sequence similarities between the viruses from patients and ticks confirm that the infections were acquired from Kotka archipelago. This finding suggests that TBEV-Eur and TBEV-Sib co-circulate in Kotka archipelago in I. ricinus ticks and raises concern for further spread of TBEV-Sib in this tick species, which is widespread in Europe. The coexistence of 2 virus subtypes and the potential emergence of more pathogenic variants requires further surveys of TBEV epidemiology and consideration of vaccination guidelines.
Acknowledgments
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We analyzed blood samples from infants born with microcephaly and their mothers in Guinea-Bissau in 2016 for pathogens associated with birth defects. No Zika virus RNA was detected, but Zika virus IgG was highly prevalent. We recommend implementing pathogen screening of infants with congenital defects in Guinea-Bissau.

In 2016, the health authorities in Guinea-Bissau reported 4 cases of Zika virus infection and 5 cases of microcephaly (I) to the World Health Organization. The Zika virus strain detected in Guinea-Bissau was the African strain (I) originally detected in Africa in 1947 and in Portuguese Guinea (now Guinea-Bissau) during 1964–1965 (2). As of March 2018, the Asian strain, which has spread throughout the Americas and Cape Verde (2) and is linked to microcephaly and other congenital abnormalities, has not been reported in Guinea-Bissau (3), and the African Zika virus strain has not been linked with microcephaly.

We report an in-depth investigation of pathogens commonly associated with birth defects in 15 infants born with microcephaly in Guinea-Bissau in 2016. Field epidemiologists identified cases of microcephaly through reports from health center personnel across the country and surveillance at Hospital Nacional Simão Mendes in Bissau, Guinea-Bissau (which has 6,000 births/y). Most cases were found in the northern and eastern regions (Gabú, Bafatá, and Oio) of Guinea-Bissau (online Technical Appendix Tables 1, 2, https://wwwnc.cdc.gov/EID/article/24/5/18-0153-Techapp1.pdf). Blood samples were collected from the mothers (median age 22 years, range 15–31 years) and infants (median age 5 months, range 1 day–9 months) and sent to Statens Serum Institut (Copenhagen, Denmark) for analysis. Three infants died before sampling, and 1 sample was lost during transport; hence, we analyzed blood samples from 11 of the 15 infants with microcephaly. For comparison, we also analyzed blood samples from 10 mothers (from Tantam Cossé, Bafatá region) of infants born without microcephaly (M.W. Rosenstiehre, unpub. data). We assayed for Zika virus and TORCH pathogens (Toxoplasma

Zika Virus IgG in Infants with Microcephaly, Guinea-Bissau, 2016

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1These senior authors contributed equally to this article.
gondii, other [Treponema pallidum, varicella-zoster virus, parvovirus B19], rubella virus, cytomegalovirus [CMV], and herpes simplex virus) (online Technical Appendix Tables 1, 2) because these pathogens are most commonly associated with congenital anomalies (4,5).

Zika virus IgG immunofluorescence assay and Zika virus neutralization test (6,7) results revealed that 14 (93%) of the 15 mothers of infants with microcephaly had Zika virus neutralizing antibodies (NAbs) (online Technical Appendix Tables 1, 2) versus 5 (50%) of the 10 mothers of healthy infants (data not shown). We tested blood samples from the 11 infants with microcephaly for Zika virus NAbs, and all were positive (presumably maternal antibodies) (online Technical Appendix Tables 1, 2). We did not perform this assay with samples from the healthy infants. No samples were positive for Zika virus RNA or IgM or had cross-neutralizing antibodies to dengue virus. Thus, the Zika virus seroprevalence among Guinea-Bissau women was surprisingly high and significantly higher in the mothers of infants with birth defects (p = 0.02 by Fisher exact test). However, timing of the Zika virus infection and strain could not be determined.

Because of sample volume limitations, we tested only 10 of 15 mothers for TORCH antibodies and all 11 infants with birth defects and available blood samples for TORCH pathogen nucleic acids (online Technical Appendix Tables 1, 2). Four infant blood samples were positive for CMV DNA and IgG but only 2 were positive for CMV IgM (online Technical Appendix Tables 1, 2). Two of these infants’ mothers were CMV IgG positive (the other 2 were not tested), and 1 mother tested positive for CMV IgM. Because sampling of infants was mainly performed 5 months postpartum rather than during the first 2–3 weeks postpartum (5,8), determining whether the CMV infections were congenital or acquired perinatally or postnatally (e.g., through breast milk) was not possible.

The mother whose infant died 5 days after birth was positive for Toxoplasma IgG (online Technical Appendix Tables 1, 2). However, samples from this child were not collected for analysis, so we could not determine whether the infant died of severe congenital toxoplasmosis. As expected, almost all mothers were positive for antibodies against parvovirus (70%), varicella-zoster virus (90%), rubella virus (90%), CMV (90%), and herpes simplex virus (100%).

Although we found a high prevalence of Zika virus NAbs and TORCH antibodies in mothers and infants, the late sampling of infants and lack of Zika virus RNA–positive samples precludes determination of the cause of microcephaly in these infants. On the basis of our findings, we propose implementing prospective surveillance in Guinea-Bissau for infants with easily identifiable congenital anomalies, such as microcephaly (i.e., head circumference 2 standard deviations below average for age and sex) (9), microphthalmia, and hearing loss, and screening these infants for Zika virus and TORCH by using blood, saliva, and urine samples collected immediately or within the first 2–3 weeks after birth. The low prevalence (0.6%) of microcephaly reported in 2015 (10) makes this suggestion feasible in resource-poor countries. If the Asian Zika virus strain is detected in Guinea-Bissau, screening of pregnant women during their first trimester should also be implemented. However, the 2-step surveillance and screening model can be applied in countries without reported detection of the Asian Zika virus strain.

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References
Heterogeneous and Dynamic Prevalence of Asymptomatic Influenza Virus Infections

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To the Editor: We read with interest the article by Furuya-Kanamori et al. on the proportion of influenza virus infections that are asymptomatic or subclinical (1), and we are troubled by a series of fundamental flaws and errors. We were concerned that the authors presented pooled estimates of the asymptomatic fraction, given the massive heterogeneity in estimates (I² values of 97%–98% in Table 1). It is not considered good practice to present pooled estimates in instances of massive heterogeneity (2). We were very surprised that the authors included volunteer challenge studies because it is well known that the severity of these infections can be modulated by the route of administration and possibly the infectious dose. We also were surprised that human infections with avian influenza viruses were included because the epidemiology of these infections differs markedly from that of human influenza viruses. These studies were mistakenly labeled as studies of pandemic influenza in online Technical Appendix 1 Table 1 (https://wwwnc.cdc.gov/EID/article/22/6/15-1080-Techapp1.pdf). When reviewing serologic studies, the authors did not define a specific antibody titer threshold but relied on the choices made in individual studies; studies that inferred influenza virus infections based on low postepidemic hemagglutination-inhibition titers, such as 10 or 20, may lack specificity because some persons could have preexisting antibodies (3). Measurement error can also be a concern. The authors probably should have excluded such studies.

In another systematic review of the asymptomatic fraction of influenza virus infections (4), we found that study designs could explain a great deal of heterogeneity in the asymptomatic fraction in studies such as outbreak investigations that used molecular testing to confirm influenza virus infections rather than serologic studies that used antibody titer measurements to indicate infections. Asymptomatic fractions were higher in general, and much more heterogeneous, in studies that followed the latter approach.

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In Response: We thank Leung and Cowling (1) for taking time to comment on our article (2). One problem with the random effects model is the rapid decline in performance of the model as the heterogeneity within studies increases. Extensive heterogeneity for asymptomatic (I² = 97%; T² = 0.31) and subclinical (I² = 97%; T² = 0.45) infection was identified. However, the model selected to pool the prevalence estimates—inverse variance heterogeneity—maintains its coverage at the nominal level, even when large heterogeneity is present (3).

Regarding inclusion criteria, we elected to review all publications detailing asymptomatic influenza prevalence in humans, as is made clear from the original article’s title onward. This method included experimental studies, as well as newly emerging zoonotic strains. We note further that the 2 experimental studies in our review had subclinical influenza infection levels within the range identified in the pooled estimate of the meta-analysis (43.4%, 95% CI 25.4%–61.8%).

Also, because antibody titers can vary drastically with technique used and between laboratories, we used the antibody titer threshold defined by each individual study.

The results/conclusions from the study published by Leung et al. (4) cannot be compared with those reported in our meta-analysis (2) for 2 important reasons. First, the case definition for asymptomatic was different; Leung et al. grouped patients without signs and symptoms at all (4) but only 1.6% had no symptoms at all (3). Second, the number of studies included in the 2 meta-analyses was different: our comprehensive review comprised 55 studies, whereas Leung et al. included a subset of only 30 studies pertaining specifically to seasonal influenza. The different studies included and different meta-analytical methods unsurprisingly yielded different outcomes.

References

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Mycobacterium lepromatosis
Lepromatous Leprosy in US Citizen Who Traveled to Disease-Endemic Areas

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To the Editor: The article by Virk et al. (1) highlighted that a person can acquire Mycobacterium lepromatosis infection without exposure to a person infected with leprosy or to known vectors during short stays (2 trips of 7 days each over 3 calendar years) in Mexico. The authors then concluded that M. lepromatosis lepromatous leprosy is a travel-related hazard for travelers to Mexico or other disease-endemic areas. We note that the exact source of acquiring the M. lepromatosis infection by the patient in this study was entirely uncertain, and experimental evidence was not enough to prove M. lepromatosis to be a travel-related hazard.

In contrast, Jessamine et al. (2) reported M. lepromatosis infection and leprosy-like illness in a patient in Canada who had no history of contact or travel to leprosy-endemic areas. Jessamine et al. indicated that transmission dynamics of M. lepromatosis infection is complex, and undiscovered mechanisms or unknown reservoir interactions may exist in such areas of nonendemic regions. Previous studies have also reported the roles of subclinical cases and environmental reservoirs in the transmission of leprosy (3,4). However, Virk et al. have not disentangled other possible sources (existence of unrecognized subclinical cases, contact with
hidden leprosy cases, and environmental reservoir) of prolonged exposure of *M. lepromatosis* to the study patient in his vicinity. Thus, the assertion of Virk et al. (1) that United States citizens can acquire *M. lepromatosis* when traveling to Mexico or other leprosy-endemic areas as tourists is misleading and demands extensive research to prove it.

In addition, it is intriguing to note that host genetic determinants can influence the acquisition and onset of leprosy (5). Therefore, the inference of a single case study cannot be generalized for all citizens of the United States. The data from these reports suggest that the epidemiologic studies of leprosy in nonendemic areas should consider travel history to delineate this issue.

References


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Spread of Plague by Respiratory Droplets or Ectoparasites

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To the Editor: Drancourt and Raoult (1) have emphasized the risk of overestimation of pneumonic plague contagion by respiratory droplets and hypothesize that only transmission of *Versinia pestis* by ectoparasites, such as lice and fleas, by close contact with infected humans can sustain outbreaks and epidemics. The outbreak of pneumonic plague in Madagascar in 2017 (2) reminds us that plague remains a potential serious threat in locations that are relatively inaccessible or have limited capacity for a robust public health response. Records describe substantial outbreaks of pneumonic plague (3) but portray a more dangerous disease than that described by Drancourt and Raoult. High rates of transmission are possible (4) when pneumonic plague is spreading through social networks, in a way similar to that observed in West Africa during the recent epidemic of Ebola virus disease (5). The Ebola virus is not thought to be easily transmitted but is clearly capable of generating a sustained epidemic.

The role of ectoparasites in the transmission of *Y. pestis* should not be dismissed. However, until a substantial epidemic has been documented with this proven etiology, this explanation of plagues, both historical and modern, must remain in the realm of conjecture.

References


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Potentially Same Novel Ehrlichia Species in Horses in Nicaragua and Brazil

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To the Editor: In our previously published report, we found that blood samples from 4 naturally infected horses in Nicaragua were PCR positive for the 16S rDNA, sodB, and groEL genes of an Ehrlichia species (1). Similarly, Vieira and colleagues reported a potentially novel Ehrlichia sp. infecting horses in South America, with a high seroprevalence in carthorses; 1 horse blood sample was PCR positive for Ehrlichia 16S rDNA and dsb genes (2). Because these 2 studies sequenced different 16S rDNA regions, the Ehrlichia sp. found in Nicaragua could not be established as the same one infecting horses in Brazil.

We retrieved an Ehrlichia PCR-positive horse blood sample (2) from Brazil and performed partial PCR and sequencing of the 16S rDNA, sodB, and groEL genes (1). Phylogenetic analysis of the sequences (3–5) demonstrated a close relationship between the Ehrlichia spp. found in Brazil and Nicaragua, with posterior probability values of 100% for all 3 gene fragments (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/24/5/17-2076-Techapp1.pdf). The 16S rDNA were 100% identical (579 bp/567 bp; GenBank accession nos. MG385129, KJ434178), sodB 99% identical (561 bp/567 bp; GenBank accession nos. MG385129, KJ434180), and groEL 99% identical (579 bp/584 bp; GenBank accession nos. MG385128, KJ434179). When we compared translated amino acid sequences of the Ehrlichia spp. from Brazil and Nicaragua, we observed high percent age identities with the groEL (100%) and sodB (97.8%) alignments (online Technical Appendix Figure 2). Furthermore, when compared with E. ruminantium, the most closely related Ehrlichia sp. on the basis of phylogenetic analyses, percent age identities from the groEL (94.8%) and sodB (78.8%) alignments were lower for both Ehrlichia spp.

These findings suggest that the novel Ehrlichia spp. found infecting horses in Nicaragua and Brazil are potentially the same species. Future studies are needed to determine cell culture practices, characterize potential clinical signs of infection, and establish the main vector of this novel equine Ehrlichia species.

References

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Human Infection with Burkholderia thailandensis, China, 2013

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To the Editor: We read with interest the research letter from Chang et al. (1). To have such severe clinical disease attributed to Burkholderia thailandensis infection
published in peer-reviewed literature is of major significance to the research community, especially given the biosecurity aspects regarding melioidosis. We are writing, however, because we have serious doubts about the identity of the organism described.

The clinical features of the case are typical of septicaemic melioidosis with pulmonary involvement. The pictures of the colonies in the technical appendix look very similar to *B. pseudomallei*, with which we have extensive experience in both Australia and Southeast Asia over the past 30 years. This identification was the most likely suggested by the phenotypic tests used. Furthermore, the virulence of the strain in mice bore more resemblance to that of *B. pseudomallei* than *B. thailandensis*, and the strain contained putative virulence determinants not normally found in *B. thailandensis*. Species identification thus appears to rest on arabinose assimilation and 16S rDNA sequence. Assimilation tests are notoriously difficult to read, and without knowledge of the 16S rDNA primers or sequence region of comparison, it is plausible that a lack of resolution between *B. pseudomallei* and *B. thailandensis* has led to incorrect species attribution.

We therefore believe that there is insufficient evidence to prove that this case was caused by *B. thailandensis* and that the presented data suggest that this isolate was, in fact, *B. pseudomallei*. We are always happy to advise colleagues about the investigation and management of possible cases of melioidosis, but in these circumstances, we felt that it was necessary to place our concerns on record.

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The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health-related images, including high-resolution (print quality) photographs, illustrations, and videos.

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The Power of Plagues, Second Edition


A usual dictionary definition of plague (a highly infectious, usually fatal epidemic disease; a pestilence; https://www.thefreedictionary.com) differs from that used by Irwin W. Sherman in his book The Power of Plagues. To accomplish his purpose in writing this book (“to make the science of epidemic diseases—plagues—accessible and understandable”), Sherman borrows his definition from historian Asa Briggs: “Plagues are a dramatic unfolding of events; they are stories of discovery, reaction, conflict and resilience of local and administrative structures.”

This book is a history of humanity as influenced and shaped by plagues of known and unknown etiology. One of its strengths is also one of its weaknesses. To trace our journey from 4 million years ago to the present, weaving in plagues, people, microorganisms, and advances in technology, is no small feat. However, for the most part, Sherman accomplishes this goal in what is a very readable book that should appeal to a wide variety of audiences. Indeed, it should be read by every student of medicine and the health professions.

Did you know that Pharaoh’s plague and snail fever are the same disease, or that war fever and jail fever are also caused by the same microorganism? Can you name 10 famous people who had syphilis or 20 famous people who died of tuberculosis? Did you know that heroin at one time was a treatment for the very troublesome cough of tuberculosis? Along with the answers to these questions, in this book you will find how plagues shaped history from ancient times to Napoleon’s invasion of Russia to the very modern plagues of HIV/AIDS, influenza, and Lyme disease.

The art or photographic reproductions, usually placed at the beginning of a chapter, are a most powerful method of connecting the reader to what life was like at a given time in history. For example, look at Figure 6.1, Eugen Le Roux’s engraving of Napoleon’s troops in Vilna after the Russian Campaign in 1812, or Figure 5.1, a photograph of Lorraine, age 11, who has AIDS, being comforted by her grandmother.

Errors in the first edition, noted by Rigau-Perez (1), have been corrected. The placement of AIDS in Chapter 5 (A 21st Century Plague, AIDS), immediately after the chapter on the Black Death, is out of order chronologically and disrupts the history timeline. Chapters 10 (Preventing Plagues: Immunization) and 11 (The Plague Protectors: Antisepsis to Antibiotics) could easily be deleted in favor of more detail or artwork. However, these criticisms are minor. The major strength of this book is that it is a very readable history of humanity as shaped by plagues, making it attractive to a wide audience.

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Reference


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Catching Breath: The Making and Unmaking of Tuberculosis

Kathryn Lougheed; Bloomsbury Sigma, New York, NY, USA; ISBN-13: 978-1472930330 (hardcover); 978-1472930347 (paperback); 978-1472930361 (Kindle); Pages: 272; Price: $27.00

Tuberculosis (TB) is the leading infectious disease cause of death worldwide, yet many persons in industrialized countries think TB is mainly a historical curiosity or a rare disease only affecting a few uniquely vulnerable persons. This paradox is the inspiration for Kathryn Lougheed’s book, Catching Breath: The Making and Unmaking of Tuberculosis. After spending a decade at the bench exploring the mycobacterium as a microbiologist, the author embraced broader historical questions and embarked on a personal pilgrimage, as if learning about the disease for the first time. The result is, in her own words, a “whistle-stop tour through the past few million years to pick apart what has made TB ‘TB’.”

Why is Mycobacterium tuberculosis still a dominant pathogen? Lougheed attempted to answer this question by exploring the ancient origins of mycobacterial infections and the expanding deadly toll of tuberculosis as human settlements grew many centuries ago. The scientific biography delves into the discovery of the microorganism at the end of the 19th century, the development of antimicrobial drugs active against the organism in the 20th century, and novel molecular techniques to detect TB genetic material and immunologic assays that have brought ever more sophisticated response to the disease. Yet Lougheed highlights that little dent has been made in TB’s enduring impact.

Whereas the book is most detailed in describing work closest to her own, the narrative extends beyond the laboratory to provoke new ways of thinking about the science of TB and assert the relevance of the humanistic and socioeconomic sides of TB. Lougheed does not shy away from the broader themes of economic inequality, political will, stigma, and interdisciplinary teamwork that affect each individual treatment and have driven and will continue to drive the epidemic’s long-term trajectory. Dr. Lougheed has marshaled an admirable sweep of complex data into a coherent and quickly moving narrative, peppering personal anecdotes throughout and bringing scenes to life. The language feels fresh, conversational, and opinionated, yet the explanations are grounded in sound research and are particularly detailed in areas related to microbiology. Her findings are often entertaining, startling, and moving.

The broad approach to such a complex topic might leave the reader wanting more clarity or depth in certain areas; however, the narrative is balanced by the enjoyment one gains from her storytelling with equal parts science, humor, history, microbiology, and real world contextualization. Readers, whether knowledgeable about tuberculosis or encountering it for the first time, will likely find that the story and the clearly posed questions deepen their interest and understanding and quicken their steps in pursuit of further answers.

Margaret J. Oxtoby, Elizabeth M. Dufort

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During the Edo period (1603–1868) in Japan, a time when Tokugawa Ieyasu and his descendants ruled, economic growth gave rise to a more literate, sophisticated, and affluent culture, hungry for the trappings of luxury, including extravagant art. This cultural shift was described by the phrase ukiyo, or “floating world,” which was associated with the pursuit of pleasure and hedonism in urban areas. The art of the Edo period, dominated by painting and woodblock prints, came to be known as “pictures of the floating world” or ukiyo-e.

Kobayashi Kiyochika (1847–1915), Mosquito Net and Full Moon at Shinagawa, 1884 (detail), from the series One Hundred Views of Musashi. Woodblock print; ink and color on paper 14 5/8 in x 9 13/16 in/ 37.2 cm x 24.9 cm). Freer Gallery of Art and Arthur M. Sackler Gallery, Smithsonian Institution, Washington, DC, United States, Robert O. Muller Collection, S2003.8.1217.

Revealing Details in Light and Shadows

Byron Breedlove and Reginald Tucker

As an artist who successfully fused motifs and ideas from Japanese and Western art, Kobayashi Kiyochika occupies a unique niche among Japanese illustrators of the Edo period. Japanese art historian, dealer, and collector Richard Lane considers Kiyochika to be both the last important ukiyo-e artist and the first exponent of the modern Japanese woodcut. Kato Yosuke, curator of Nerima Art Museum, Tokyo, explains that “Kiyochika is often referred to as the last ukiyo-e artist” because he stuck to colored woodblock prints and “kept pinning his hope on their potential until the end, despite the diversification and development of printing techniques in modern times.”

Kiyochika’s ukiyo-e color woodblock prints, as well as his newspaper illustrations and wartime propaganda art,
document the rapid modernization that occurred during the reign of Emperor Meiji (1867–1912). As Japan transitioned from being an isolated shogunate state to becoming an imperial world power, it experienced an industrial revolution and opened its ports and cities to other countries. Yosuke wrote that Kiyochika “must have had the pride of a defeat-ed person because he was a vassal of the shogun born in Edo (present-day Tokyo).”

Miriam Wattles, professor of art at the University of California Santa Barbara, who specializes in Japanese visual art, sees distinctive influences from both Western and Japanese cultures in Kiyochika’s prints. Wattles states that his series of One Hundred Views of Musashi, which includes this month’s cover image, Mosquito Net and Full Moon at Shinagawa, “appropriates perspective, format, and style from Katsushika Hokusai, the most prolific and recognized Japanese artist of the late Edo period.” Wattles and others suggest that English painter Charles Wirgman, who in 1861 went to Japan on assignment as the visual reporter for London Illustrated News, influenced and may have briefly instructed the print maker.

This print displays the artist’s mastery of kösen-ga, or “pictures of sunbeams,” a technique that portrays the interplay of light and shadows. Mosquito Net and Full Moon at Shinagawa harbor appears a deviously simple subject, but Kiyochika’s composition, rendered in a photographic perspective, is brimming with details and contrasts that invite close scrutiny and calmness.

The edge of the draped mosquito net bisects a full moon; its mesh diffuses the moonlight and slices the moon into a yin and yang of darkness and light. The shimmering moonlight reflects on the surface of the water, and the artist contrasts not only the interplay of light and dark, but offers two views: one unobstructed, one through the netting. While several smaller boats drift near the horizon, Kiyochika placed a solitary sailboat near the center of the print, passing across the view and visible through the netting. Carefully etched vertical lines define the bamboo structure and contrast with the horizontal rippling shadows and reflections of the water’s surface. A jutting tree limb reaches over the water, yet no land is visible. There is a tactile texture, too. It’s easy to imagine the tautness of a paper lantern, the washboard surface of a bamboo wall, or the feel of a gauzy mesh mosquito net.

Although mosquito nets were introduced to Japan from China as early as 720, during the Edo period, silken nets were desired as luxury items and were widely used well into the 20th century, until glass windows and doors, air conditioning, and pesticides became commonplace. Mosquito control with long-lasting insecticidal nets continues to offer simple, inexpensive, but not infallible protection to people in many parts of the world. However, a confluence of demographic, environmental, and societal factors are enabling mosquitoes and other vectors that spread disease-causing pathogens to expand their territories. Developing diverse integrated, innovative approaches to use alongside those measures that remain effective and to replace those that are no longer effective at controlling the spread of vectorborne diseases remains a critical public health imperative.

Bibliography


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Emerging Infectious Diseases

Upcoming Issue

- Absence of Nosocomial Transmission of Imported Lassa Fever during Use of Standard Barrier Nursing Methods
- Multistress-Tolerant *Campylobacter jejuni* Frequently Implicated in Human Infection
- Hepatitis E in Long-Term Travelers from the Netherlands to Subtropical and Tropical Countries, 2008–2011
- Occupation-Associated Fatal Limbic Encephalitis Caused by Variegated Squirrel-1 Bornavirus, Germany, 2013
- Influenza D Virus Infection in Feral Swine Populations, United States
- Bioclinical Score to Predict Nephropathia Epidemica Severity at Hospital Admission
- Foot-and-Mouth Disease Caused by an A/ASIA/G-VII Virus Lineage, Middle East, 2015–2016
- Novel Poxvirus in Proliferative Lesions of Wild Rodents in East-Central Texas
- Importation of Human Seoul Virus Infection to Germany from Indonesia
- Mixed *Leptospira* Infections in Diverse Reservoir Host Community in Madagascar
- Marburg Virus Infection in Egyptian Rousette Bats, South Africa
- *Brucella suis* Infection in Dog Fed Raw Meat, the Netherlands, 2016
- *Ehrlichia muris* in *Ixodes cookei* Ticks, Northeastern United States, 2016–2017
- Novel Parvovirus Related to Primate Bufaviruses in Dog
- Novel *Salmonella enterica* Serovar Typhimurium Genotype Levels as a Predictor of Seasonal Salmonellosis Epidemics
- Westward Spread of Highly Pathogenic Avian Influenza A (H7N9) Virus among Humans, China
- Use of Bead-Based Serology Assay to Evaluate Chikungunya Epidemic in Haiti
- Genomic Sequencing of *Bordetella pertussis* for Epidemiology and Global Surveillance of Whooping Cough
- Genomic Epidemiology of Global Carbapenemase-Producing *Enterobacter* spp., 2008–2014

Complete list of articles in the June issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

May 6–9, 2018
ASM Clinical Virology Symposium
West Palm Beach, FL, USA

June 7–11, 2018
ASM Microbe
Atlanta GA, USA
https://www.asm.org/index.php/asm-microbe-2018

June 21–22, 2018
Emerging Diseases International Meeting
Institut Pasteur
Paris
http://www.emergingdiseases.conferences-pasteur.org/home

August 26–29, 2018
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA
https://www.cdc.gov/iceid/index.html

September 23–26, 2018
ASM Conference on Rapid Applied Microbial Next-Generation Sequencing and Bioinformatic Pipelines
Tysons, VA, USA
https://www.asm.org/

Oct 1–3 2018
International Conference on Migration Health
Sponsored by the International Society of Travel Medicine
Rome, Italy
http://www.istm.org/ICMH2018

October 3–7, 2018
ID Week
San Francisco, CA, USA
http://www.idweek.org/

October 28–30, 2018
2018 Annual Congress
International Society for Vaccines
Atlanta, GA, USA

Announcements
Email announcements to EIDEditor (eideditor@cdc.gov). Include the event’s date, location, sponsoring organization, and a website. Some events may appear only on EID’s website, depending on their dates.
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 http://www.medscape.org. If you are not registered on http://www.medscape.org, please click on the “Register” link on the right hand side of the website.

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 this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.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 go to https://www.ama-assn.org. 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 AMA PRA CME credit certificate, and present it to your national medical association for review.

Article Title
Surveillance for Mosquitoborne Transmission of Zika Virus, New York City, NY, USA, 2016

CME Questions

1. You are advising a public health department regarding the potential for locally acquired, mosquito-borne Zika virus infections. Based on the New York City Department of Health and Mental Hygiene (NYC DOHMH) surveillance study by Wahnich and colleagues, which one of the following statements about surveillance findings in New York City from June to October 2016 is correct?
   A. Of 15 suspected cases reported and tested by urine reverse transcription PCR, 12 were negative
   B. There were 308 emergency department (ED) visits for Zika-like illness, 40,073 visits for fever, and 17 unique spatiotemporal clusters of ED visits for fever
   C. Sentinel surveillance suggested local mosquitoborne Zika virus transmission
   D. Enhanced passive surveillance suggested local mosquitoborne Zika virus transmission

2. According to the NYC DOHMH surveillance study by Wahnich and colleagues, which one of the following statements about possible surveillance tools for jurisdictions concerned about the possibility of local mosquitoborne Zika virus or other arboviral transmission is correct?
   A. 76% of sentinel sites reported suspected cases, which suggests a high level of engagement at most sites
   B. All hospitals had completely transitioned to HL7 international reporting standards, facilitating syndromic surveillance
   C. Future sentinel surveillance programs are unlikely to benefit from an evaluation component
   D. Enhanced passive surveillance was an efficient, manageable tool to monitor for locally transmitted mosquitoborne Zika virus

3. On the basis of the NYC DOHMH surveillance study by Wahnich and colleagues, which one of the following statements about other clinical and public health implications of this surveillance study regarding mosquitoborne Zika virus infection would be correct?
   A. For sentinel surveillance, the use of urine for Zika virus testing is not recommended
   B. Median time between specimen collection and result availability was 72 hours, suggesting undesirable delays
   C. Continued collaboration between DOHMH and EDs to improve travel data will facilitate surveillance for Zika virus and for other travel-associated diseases
   D. Fever syndrome alone proved to be the best choice for case detection
**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.

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**Article Title**

**Two Cases of Israeli Spotted Fever with Purpura Fulminans, Sharon District, Israel, 2016–2017**

**CME Questions**

1. Which of the following subspecies of *Rickettsia conorii* is associated with the highest case fatality rate among humans?
   - A. *R. conorii* subsp. *conorii*
   - B. *R. conorii* subsp. *caspia*
   - C. *R. conorii* subsp. *indica*
   - D. *R. conorii* subsp. *israelensis*

2. Which of the following symptoms or complications is most likely to be part of the presentation of Israel spotted fever (ISF)?
   - A. Maculopapular rash involving the palms and soles
   - B. Endocarditis
   - C. Endophthalmitis
   - D. Purpura fulminans

3. Which of the following history or signs was most common among the 5 cases of ISF presented in the current study?
   - A. Known exposure to ticks
   - B. Transaminitis
   - C. A single eschar
   - D. Diarrhea

4. Which of the following tests was most helpful in the early identification of rickettsial disease in the current case series?
   - A. Serum immunoglobulin M for spotted fever group rickettsia
   - B. Serum immunoglobulin G for spotted fever group rickettsia
   - C. The presence of leukocytosis
   - D. Skin biopsy polymerase chain reaction tests
Available Now - New for 2018

The fully revised and updated CDC Yellow Book 2018: Health Information for International Travel codifies the U.S. government’s most current health guidelines and information for clinicians advising international travelers, including pretravel vaccine recommendations, destination-specific health advice, and easy-to-reference maps, tables, and charts.

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- New cholera vaccine recommendations
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Class of 2017
JOURNAL BACKGROUND AND GOALS

What are “emerging” infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as “emerging.” These diseases, which respect no national boundaries, include:

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an “Emerging” Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC’s efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC’s efforts against the threat of emerging infections. However, even as it addresses CDC’s interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

- Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
- Reports laboratory and epidemiologic findings within a broader public health perspective.
- Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

- Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
- Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
- Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.
Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Summary of Authors’ Instructions

Authors’ 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 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 scripts. Number citations in order of appearance (including in text, figures, and tables).

Authors if only two. Include affiliations and the author’s primary research interests.

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 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”).

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 50 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 in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 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 50 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.

Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article’s publication.

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.

Video Reports. Video reports 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.

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. Etymologica (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.
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