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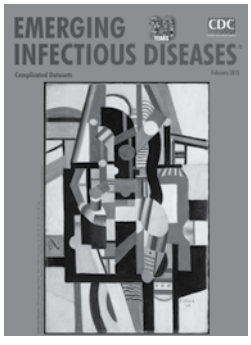

On the Cover

***Mechanical Elements*, 1920**

Fernand Léger (French, 1881–1955)

Oil on canvas; 36 1/8 x 23 1/2 in (91.8 x 59.7 cm)

Metropolitan Museum of Art
Jacques and Natasha Gelman Collection, 1998 (1999.363.36)

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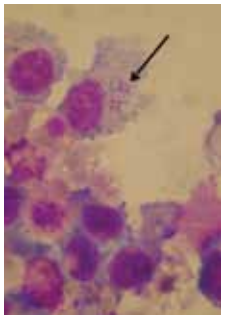

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Online Report

Melioidosis Diagnostic Workshop, 2013
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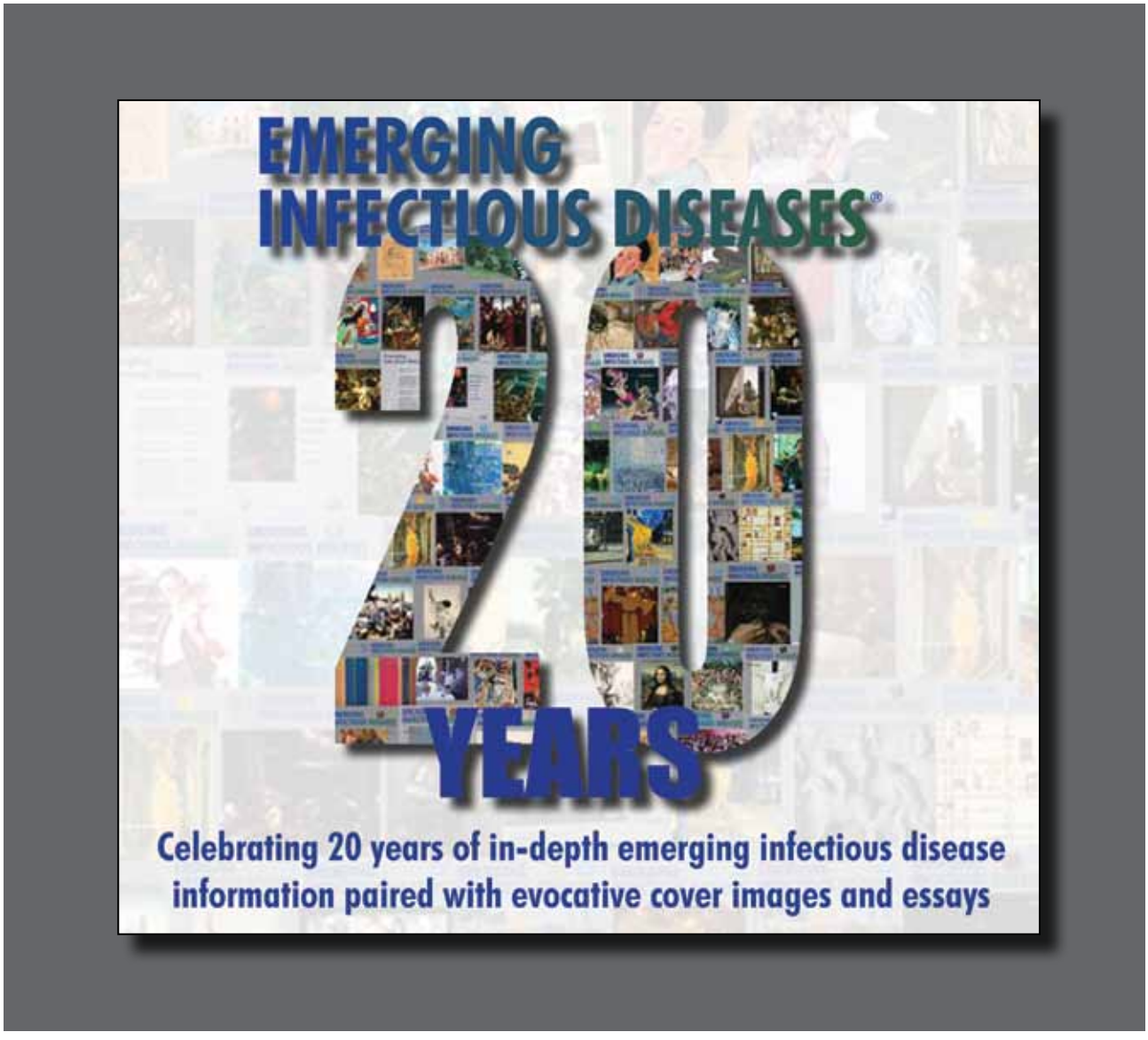

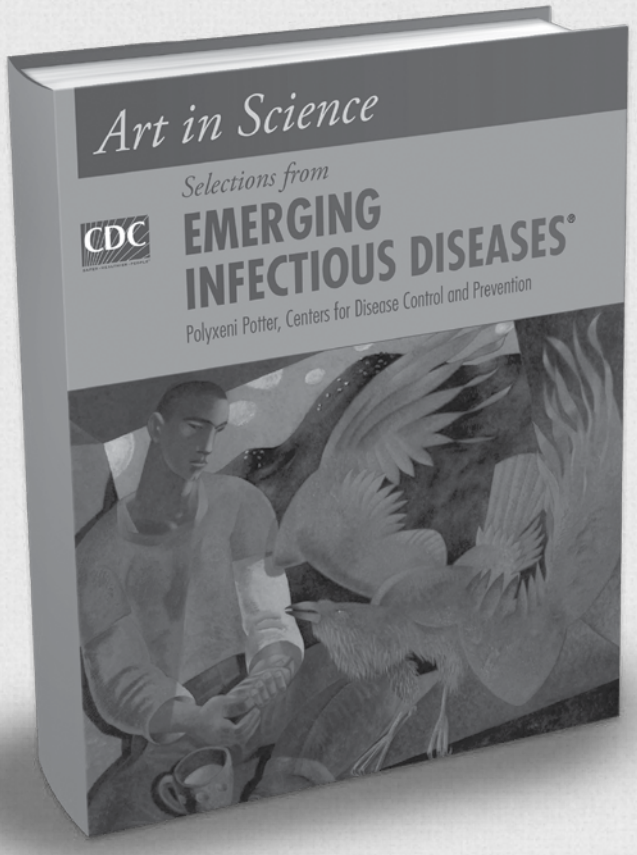

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Entry Screening for Infectious Diseases in Humans

Linda A. Selvey, Catarina Antão, Robert Hall

In response to the severe acute respiratory syndrome (SARS) pandemic of 2003 and the influenza pandemic of 2009, many countries instituted border measures as a means of stopping or slowing the spread of disease. The measures, usually consisting of a combination of border entry/exit screening, quarantine, isolation, and communications, were resource intensive, and modeling and observational studies indicate that border screening is not effective at detecting infectious persons. Moreover, border screening has high opportunity costs, financially and in terms of the use of scarce public health staff resources during a time of high need. We discuss the border-screening experiences with SARS and influenza and propose an approach to decision-making for future pandemics. We conclude that outbreak-associated communications for travelers at border entry points, together with effective communication with clinicians and more effective disease control measures in the community, may be a more effective approach to the international control of communicable diseases.

Many countries instituted border screening in response to the severe acute respiratory syndrome (SARS) pandemic of 2003 and the influenza A(H1N1)pdm09 virus pandemic of 2009, and although not formally evaluated, the experiences of several countries have been documented (*1*–*11*). Given the recent emergence of the influenza A(H7N9) virus in many parts of China (*12*), Middle East respiratory syndrome coronavirus in Saudi Arabia (*13*), and the current, most widespread Ebola outbreak in Africa (*14*), it seems timely to consider the costs and the effectiveness of border screening, as shown by recent experiences. Herein, we discuss the use of border-screening measures instituted during the 2003 SARS pandemic and the 2009 influenza pandemic.

Border screening, together with isolation of persons identified with suspected cases of disease and quarantine of their contacts, is implemented to delay or prevent the entry of infected persons to a country/geographic area or to prevent the global spread of a disease from a source country. The intent of border screening is to detect possibly infectious persons at the border, either on entry to or exit from a country, so that they can be placed in isolation or prevented from traveling and spreading the disease elsewhere; however, this strategy is useful only if the intended goal is successfully achieved. Other potential benefits of border screening relate to increasing public awareness about and confidence in protection from the disease in question, but the scope of this article does not allow for a discussion of these benefits.

During the 2009 influenza A(H1N1)pdm09 virus pandemic, the World Health Organization advised persons who were ill with influenza to delay travel (*15*). Early during the SARS pandemic and in August 2014 during the Ebola virus epidemic, the World Health Organization recommended border exit screening of travelers from affected countries (*16*,*17*). Border screening can be undertaken through self-identification by means of health declaration cards, airline/transit agency notification to health authorities of sick passengers, visual inspection of travelers, and/or fever screening of travelers implemented through the use of infrared thermal image scanners (ITISs). Three key questions are the following: How effective have these measures been at detecting ill travelers? Are there situations in which border screening is likely to be effective? If border screening is not effective, are there any other measures that could be implemented to prevent the spread of disease beyond the source country? To explore these questions, we examined border-screening experiences during the influenza A(H1N1)pdm09 virus pandemic and the SARS pandemic. Questions relating to the effectiveness of border screening are relevant regardless of the situation in which they are applied, including limited screening from one part of the world or screening on isolated island countries, because the experiences relate to the effectiveness of the measure itself in detecting cases at the border.

Border Screening and the Influenza A(H1N1)pdm09 Virus Pandemic

Because of a short incubation period and consequent short serial interval (i.e., time between the onset of the first case and the onset of subsequent case[s]), influenza virus causes explosive outbreaks despite its relatively low infectivity. Influenza A(H1N1)pdm09 virus, which spread rapidly throughout the world in 2009, was most likely established in Australia (*18*) and Japan (*19*) before border screening was initiated in those countries. Border screening to detect

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influenza-infected travelers is likely to be unsuccessful because persons with asymptomatic cases can be infectious, and fever is not a consistent symptom of influenza (*20*). This means that screening sensitivity is low and a substantial proportion of infectious persons will not be detected at the border, and those that are detected may well have transmitted the virus to other persons before being isolated. This was the experience of several countries during the influenza A(H1N1)pdm09 virus pandemic. For example, in Singapore, of the first 116 influenza A(H1N1) pdm09 virus–infected persons identified with a history of recent international travel, only 15 (12.9%) were identified through screening at the airport (*2*). In Japan, intensive border screening was in place at the main international airport during April 28–June 18, 2009. Of 151 influenza cases that might have been acquired during travel overseas, only 10 (6.6%) were detected as a result of border screening in Japan (*4*). During the same period in New South Wales, Australia, an estimated 6.7% (3/45) of imported cases were detected at the border (*9*), and in Auckland, New Zealand, 5.8% (4/69) of the cases were detected at the airport (*10*). Singapore, Japan, and Australia, but not New Zealand, used ITISs to screen for fevers at their borders, even though the sensitivity of this screening was similarly low at the sites.

Before the influenza A(H1N1)pdm09 virus pandemic, a modeling study suggested that the use of thermal scanners at airports/entry points to screen incoming passengers or at exit points from countries where influenza virus is circulating could reduce the number of cases that would otherwise occur during a pandemic (*21*). However, the study assumed a 50% detection rate for all incoming infected persons, including those with asymptomatic cases and those incubating the virus (*21*). In practice, detection was substantially lower than that.

ITISs were used in many countries to detect febrile passengers. A review of hospital-based studies examining the efficacy of ITISs in detecting fever found that the sensitivity of fever detection ranged from 4% to 89.6%, and the positive predictive value with a 1% prevalence of fever ranged from 3.5% to 65.4% (*22*). A more recent study involving airline travelers estimated a positive predictive value of ITIS for fever detection of 0.9%–4.1% for detecting fever of any cause and a positive predictive value of fever for influenza of 2.0%–2.8% for detecting influenza-associated fever (*3*). Therefore, many persons with possible fever would have to be identified before a case of influenza was detected, and screening for fever is unlikely to be sensitive enough to detect sufficient numbers of influenza cases to prevent or slow the importation of a pandemic strain.

Several other models have assessed the role of travel restrictions on the international spread of influenza (*23*–*25*). These models concluded that unless travel restrictions prevented >99% of travel, they would, at best, delay the introduction of pandemic influenza by 2–3 weeks, and because of the explosive nature of the epidemic, would have no overall effect on the total number of cases (*23*–*25*). The results are effectively the same whether travel restrictions are used (as in these models) or screening and isolation/quarantine are used to limit the movement of possibly infectious persons. However, the conclusion from these models (i.e., that allowing only a small number of cases to enter a country would result in an epidemic of the same size as if travel restrictions were not in place) is applicable to screening. It is probable that entry screening with a low rate of detection of incoming cases would also be unlikely to significantly delay the commencement of an epidemic or reduce the total number of cases. The models had also not been validated using data from an influenza pandemic (*26*). Now that data from the influenza A(H1N1)pdm09 virus pandemic are available, there is an opportunity to validate the models examine the efficacy of border measures.

A substantial amount of resources were expended on border-screening measures in several countries, including Australia. At a time when clinical and public health services were stretched in responding to the pandemic, there were major opportunity costs resulting from the application of border screening (*4*,*9*). In New South Wales, it was estimated that the cost of staffing airport clinics was $50,000 AUD/case detected (*9*).

Border Screening and the SARS Pandemic

More than 10 years have elapsed since the SARS virus emerged in China. From its emergence in November 2002 through July 2003, the virus, which has an incubation period of 2–12 days (mean 4–5), infected >8,000 persons across 30 countries (*27*). Despite the lack of antimicrobial drugs or a vaccine, the epidemic was controlled worldwide through a combination of early isolation of case-patients, quarantine of contacts, and strict infection control measures (*20*,*27*).

Fraser et al. (*20*) modeled the control of communicable diseases according to the diseases' characteristics of infectiousness during the incubation period and in asymptomatic infections. According to this model, public health measures are likely to be effective if persons are infectious only when symptomatic, particularly if infectivity peaks after the onset of symptoms. This means that infected persons are not infectious during the incubation period or during asymptomatic infection. During SARS virus infection, peak viremia (and assumed infectivity) occurs 10 days after symptom onset (*28*), and this timing coincides with the severity of symptoms. Persons with asymptomatic infection and persons in the incubation period do not appear to be infectious (*27*,*28*). Therefore if case-patients are isolated within 2–3 days of infection, transmission will be limited (*29*). If contacts are quarantined until beyond the incubation period, this will also

limit further transmission. High fever (>38°C) is a common symptom among persons seeking medical care for SARS, but case-patients with fever of <38°C or who were afebrile have been described and have been implicated in the transmission of SARS in a health care setting (*30*–*33*). This information suggests that active case finding, isolation, strict infection control, and contact tracing will limit the spread of SARS, and modeling suggests that the combination of these measures would be the most effective control strategy (*20*,*29*). This information also suggests that border measures that involve effective case detection (i.e., a high proportion of cases detected), especially if associated with opportunities for effective contact tracing (i.e., contacts quarantined within 2 days of case-patient contact), could be useful strategies for delaying the entry of SARS into a country and limiting opportunities for the virus to spread. However, the long SARS incubation period means that cases of imported disease could easily occur through the border entry of infectious, asymptomatic persons.

During the SARS epidemic, several countries instituted border measures, including travel warnings, educational information for travelers, and border screening. In Australia, Canada, and Singapore, a combination of border screening measures was instituted, yet no confirmed SARS cases were detected in any of the 3 countries (*5*–*7*). In Australia, where ITISs were not used, 4 suspected/probable SARS cases were detected at the border. Those 4 cases represented 13.8% of the 29 persons detected in Australia with suspected/probable SARS during the screening period who were symptomatic at the time of arrival in the country (*5*). Five suspected/probable SARS case-patients arrived in Canada during the screening period; symptoms developed in all 5 patients after arrival, and none of the cases were detected at the border (*6*). The authors concluded that because of the very low prevalence of infection among travelers, the positive predictive value of any border screening would be effectively zero (*6*).

Two independent modeling studies (*29*,*34*) modeled the effect of entry screening for SARS on SARS importation and subsequent spread. Glass and Becker (*29*) concluded that entry screening for SARS would not reduce the probability of an outbreak of 100 cases by >7%; this conclusion is based on the assumption of screening effectiveness equivalent to that estimated based on the Australian experience. Goubar et al. (*34*) also concluded that entry screening would play a minimal role in reducing the number of imported cases, on the basis that border screening would miss infected travelers who are currently incubating the infection (*34*). Both studies concluded that SARS transmission within a country could be more effectively limited by gearing-up health services to enable early detection and isolation of case-patients than by investing in border screening (*29*,*34*).

To Screen or Not To Screen

We do not recommend border screening at any time during the evolution of an influenza pandemic because the sensitivity and specificity of influenza screening are low, regardless of the method (e.g., self-identification, thermal scanning, and/or visual inspection). Border screening is resource intensive, and there is a significant opportunity cost for other public health measures if border screening is in place. For example, in Australia during May 2009 (i.e., during the influenza pandemic), an average of 28,685 persons arrived at 8 airports via international air flights (*1*). Entry screening was in place at the time, and each screening point with an ITIS required 1–2 operators at all times when flights were arriving. Trained nurses were required to be present at each airport at all times when there were incoming flights to provide follow-up for any passengers identified through ITIS screening or who self-identified as being unwell. An additional person was employed at each airport at all times when flights were arriving to assist with administrative activities. During April 28, 2009–June 1, 2009, a total of 15,457 (≈1.5%) airline travelers arriving at airports across Australia were identified as being unwell. Most (84%) of these persons self-identified as being unwell on health declaration cards; only 0.5% were identified by the use of an ITIS (*1*). Of these 15,457 persons, only 154 were subsequently treated as if they were infected with the pandemic influenza virus.

Influenza outbreaks are difficult to control without the use of vaccines and antiviral drugs. The public health response should focus on early identification and treatment of cases at risk of becoming severe; social-distancing measures applied at the community level; infection control measures; vaccination (when a vaccine becomes available); and in some cases, antiviral prophylaxis. Focusing on these measures instead of border screening will be more fruitful.

Compared with influenza, SARS is more amenable to border screening because fever is a more consistent symptom and infected persons are not infectious when asymptomatic or during the incubation period. However, persons who are incubating the SARS virus will not be detected by screening, and, given the low prevalence of infection even in source countries, the positive predictive value of screening will be very low. Therefore, we also do not recommend border screening for SARS. SARS is, however, amenable to control through the use of a combination of measures: early isolation of confirmed case-patients, quarantine of case-patient contacts, and strict infection control (*20*,*27*). Measures that will enable the early detection and isolation of case-patients and quarantine of contacts should be the focus of resource allocation.

Communication as a Border Measure

Communication with incoming travelers was a key component of border activity during the SARS and influenza

A(H1N1)pdm09 virus pandemics and during other disease outbreaks (*8*,*35*,*36*). Communication can take many forms, including informational videos, posters, signs, in-flight announcements, flyers, and health alert notices (HANs) (*36*). During the 2009 influenza pandemic, 44% and 84% of travelers identified as unwell on arrival in Singapore (*2*) and Australia (*1*), respectively, self-identified as being ill; this finding suggests that communication to incoming travelers can be a useful mechanism to encourage self-reporting. However, the evidence of the effectiveness of communication measures at borders is limited (*6*,*36*). Travel HANS (T-HANs) have been used in the United States by the Centers for Disease Control and Prevention (Atlanta, GA) since the 1970s as a communication tool directed to incoming travelers. T-HANs provide travelers with information about a current disease outbreak, symptoms of the disease, and advice about seeking medical care should symptoms occur. T-HANs also include clinical guidance and resources for physicians. Selent et al. (*35*) evaluated the effectiveness of T-HANs in encouraging the self-identification and health care–seeking behavior of incoming travelers from Haiti during the cholera epidemic in that country in 2010. The evaluation suggested that the T-HANs provided a small positive influence on health care–seeking behavior among incoming travelers (*35*). The use of current communication technologies (e.g., the Internet or short text messages to mobile phones) could also be investigated. SMS (short message service) messages, for example, have been used successfully in other areas of public health (*37*).

The use of T-HANs and other communication methods is a potentially worthwhile border measure that could assist with the early identification and appropriate management of incoming passengers with a disease of interest. Such measures need to be accompanied by the provision of appropriate health care for travelers who are deemed ill, and must be easily understandable. As with any health communication endeavor, effective communication requires multiple modes of communication and tailored messages (*38*).

Although the provision of consistent and repeated early warnings and information about infectious disease outbreaks to local clinicians is not a border measure, it can be highly effective in supporting the rapid recognition and isolation of possibly infectious incoming travelers. This fact is exemplified by the experience of SARS in Canada, where alert clinicians in Vancouver, British Columbia, isolated a patient with SARS within 15 minutes of his/her arrival at the clinic and used appropriate respiratory protection, but clinicians in Toronto, Ontario, did not quickly isolate a patient with SARS or use adequate respiratory protection when treating the patient. Both case-patients sought care at a hospital on the same day during a time when significant SARS transmission was ongoing in Ontario but not in Vancouver. Subsequent investigation identified well-communicated and repeated warnings about SARS to local clinicians as being an important factor in limiting further spread of SARS in Vancouver (*39*).

Conclusions

Historically, most attempts at border screening have been ineffectual, as demonstrated by the pandemic spread of SARS and influenza A(H1N1)pdm09 to many countries despite the use of border screening. Modeling and observational studies have indicated that border screening is likely to be unsuccessful in preventing or delaying the entry of such diseases into a country. Border screening generally has high opportunity costs, both financially and in terms of the use of scarce public health staff resources at a time of high need. We conclude that border screening should not be used. Instead, the less costly measure of providing information to arriving travelers is recommended, together with effective communication with local clinicians and more effective disease control measures in the community.

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The views expressed in this paper are not necessarily shared by the Australian Government Department of Health.

Dr. Selvey is Director of Epidemiology and Biostatistics at the Curtin University School of Public Health. Her research interests include the epidemiology and control of communicable diseases.

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SYNOPSIS

Timing of Influenza A(H5N1) in Poultry and Humans and Seasonal Influenza Activity Worldwide, 2004–2013

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Co-circulation of influenza A(H5N1) and seasonal influenza viruses among humans and animals could lead to co-infections, reassortment, and emergence of novel viruses with pandemic potential. We assessed the timing of subtype H5N1 outbreaks among poultry, human H5N1 cases, and human seasonal influenza in 8 countries that reported 97% of all human H5N1 cases and 90% of all poultry H5N1 outbreaks. In these countries, most outbreaks among poultry (7,001/11,331, 62%) and half of human cases (313/625, 50%) occurred during January–March. Human H5N1 cases occurred in 167 (45%) of 372 months during which outbreaks among poultry occurred, compared with 59 (10%) of 574 months that had no outbreaks among poultry. Human H5N1 cases also occurred in 59 (22%) of 267 months during seasonal influenza periods. To reduce risk for co-infection, surveillance and control of H5N1 should be enhanced during January–March, when H5N1 outbreaks typically occur and overlap with seasonal influenza virus circulation.

Co-circulation of influenza A viruses in human and animal reservoirs can provide opportunities for these viruses to reassort and acquire genetic material that facilitates sustained human-to-human transmission, a necessary trait of pandemic viruses (1). One influenza strain at the forefront of pandemic preparedness planning is highly pathogenic avian influenza (HPAI) A(H5N1) virus. Asian-lineage H5N1 viruses emerged in domestic birds in Southeast Asia in 1996 and are now endemic in 5 countries (2). As of December 2013, H5N1 virus outbreaks have been documented among domestic poultry and wild birds in >60 countries (3,4). The spread of H5N1 among the world's domestic poultry population increases the risk for H5N1 to infect humans.

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Humans are at risk for H5N1 infection if they have direct or close contact with infected domestic poultry, such as by handling sick animals or their byproducts, caregiving, slaughtering, and butchering; infection can also occur through some forms of indirect contact (e.g., proximity to live poultry or wet markets) (*5*,*6*). During 2003–2013, more than 645 human cases of HPAI H5N1 were confirmed; the case-fatality rate was ≈60% (*7*). H5N1 viruses have not yet acquired the ability to be transmitted between humans beyond 3 generations, therefore failing to show sustained human-to-human transmission (*8*). However, these viruses have wide geographic distribution and the potential to reassort with human seasonal influenza viruses. These characteristics mean that clarifying the timing of H5N1 outbreaks among poultry and infections in humans may be useful for prevention and control activities.

Recently published data suggest a seasonal pattern to H5N1 virus infection among domestic and wild birds (*5*,*9*–*13*). Park et al. noted that H5N1 outbreaks among poultry and infections in humans in Southeast Asia occurred in the cooler months during 1997–2006 (*9*). Other studies from Southeast Asia have suggested that decreasing temperatures may correlate with an increase in the number of H5N1 outbreaks among birds (*14*,*15*). Although ecologic studies suggest that H5N1 virus activity may occur during predictable times of the year, a systematic analysis of global poultry and human H5N1 data has not tested this hypothesis. In this study, we explored whether H5N1 outbreaks among domestic poultry and human H5N1 cases occurred in temporal proximity, occurred during certain climate conditions, or overlapped with human seasonal influenza epidemics.

Methods

Timing of H5N1 Outbreaks among Poultry

To describe the temporal patterns of H5N1 outbreaks among poultry worldwide, we extracted the number of monthly outbreaks reported by 52 countries as immediate notifications to the World Organisation for Animal Health (OIE) during January 2004–December 2013 (*16*). We used

the reported onset month as the occurrence month for each outbreak. Egypt and Indonesia stopped submitting immediate notifications of new H5N1 outbreaks among poultry after H5N1 in poultry was declared endemic: for Egypt in July 2008, and for Indonesia in September 2006. To obtain the number of monthly outbreaks for these countries, we extracted the number from their OIE biannual disease reports. If only the number of new cases but not the number of outbreaks in which they occurred was reported, we classified the month as having 1 outbreak.

For each country and year, we designated the peak month of H5N1 activity as the month that had the largest number of reported H5N1 outbreaks among poultry. Next, we identified the average peak month of H5N1 activity for each country (e.g., if the peak months in a hypothetical country occurred during January 2010, February 2011, and March 2012, then the average peak month during this period would have been February for this country).

Timing of Human H5N1 Cases and Poultry H5N1 Outbreaks

We identified the monthly number of human H5N1 cases reported to the World Health Organization (WHO) during January 2004– June 2013. Initially, we obtained data from all 15 countries that reported human H5N1 cases since January 2004 (Figure 1). We then restricted the analysis to the 8 countries that reported 97% of all human H5N1 cases and that reported recurrent H5N1 activity among humans and poultry. To examine the temporal relationship between human H5N1 cases and H5N1 outbreaks among poultry, we compared the number of human H5N1 cases to the number of poultry H5N1 outbreaks for each month from these 8 countries (*7,17*). We then analyzed the association of the monthly number of human H5N1 cases and poultry H5N1 outbreaks by using the Spearman coefficient (ρ).

Timing of Human Seasonal Influenza and H5N1 Cases

To clarify the hypothetical risk for human co-infection with avian and seasonal influenza viruses, we investigated the extent to which human H5N1 cases occurred during months when seasonal influenza viruses were circulating. To determine whether H5N1 and seasonal influenza viruses were identified among humans during the same months, we obtained seasonal influenza surveillance data collected by WHO during 2004–2013 (*18*). We estimated periods of seasonal influenza epidemics by assessing the months during which the proportion of respiratory samples that tested positive for influenza viruses was higher than the annual mean (*19*). We then identified the number of months during which human H5N1 cases and human seasonal influenza epidemics occurred during the same month.

Ambient Temperature, Human H5N1 Cases, and Poultry H5N1 Outbreaks

To determine whether cool ambient temperature was associated with the number of poultry H5N1 outbreaks, we

Figure 1. Shading indicates countries that reported confirmed human cases of highly pathogenic avian influenza (HPAI) A(H5N1) infection and outbreaks of H5N1 among poultry during 2004–2013. Black shading indicates the 8 study countries that reported 97% of all human H5N1 cases and 90% of all poultry H5N1 outbreaks: Bangladesh, Cambodia, China, Egypt, Indonesia, Thailand, Turkey, and Vietnam.

Table. Summary data for 8 countries that reported 90% of worldwide influenza A(H5N1) outbreaks among poultry and 97% of all human H5N1 cases, January 2004–December 2013*

Country	Geographic region	No. poultry H5N1 outbreaks	No. human H5N1 cases	Peak month for poultry H5N1 outbreaks	Peak month for human H5N1 cases	Peak month for human seasonal influenza cases
China	Northern Temperate	119	44	February	January	February
Egypt	Northern Temperate	2,516	173	March	March	December
Turkey	Northern Temperate	225	12	January	January	March
Bangladesh	Northern Temperate/Tropical	549	7	February	March	July
Cambodia	Tropical	35	47	February	January	October
Indonesia	Tropical	3,555	195	February	January	December
Thailand	Tropical	1,138	25	October	January	September
Vietnam	Tropical	3,194	122	February	January	June

*H5N1 was declared endemic in Indonesia in 2005 and Egypt in 2007. After these dates, these countries did not report outbreaks to the World Organisation for Animal Health on a monthly basis. To obtain the monthly number of outbreaks after countries become endemic, we analyzed their 6-month reports; these reports are made by every country.

abstracted monthly mean temperature data for each country from the US National Oceanic and Atmospheric Administration or from the Vietnam General Statistics Office (*20*,*21*). We used linear regression models to determine whether the monthly number of poultry H5N1 outbreaks and number of human H5N1 cases were negatively associated with mean monthly temperature and precipitation (e.g., after accounting for the absolute value of each country's central latitude [*19*] and 2-way interactions) (*22*).

Results

Timing of Poultry H5N1 Outbreaks

During January 2004–June 2013, a total of 12,610 poultry H5N1 outbreaks in 52 countries were reported to the OIE. Fifteen of these countries also reported laboratory-confirmed H5N1 cases in humans to WHO. Of these 15 countries, 8 (Bangladesh, Cambodia, China, Egypt, Indonesia, Thailand, Turkey, and Vietnam) accounted for 11,331 (90%) of worldwide poultry H5N1 outbreaks. These 8 countries served as the basis for our analyses. Four of the 8 countries (Bangladesh, China, Egypt, and Turkey) are classified as Northern Temperate or Subtropical countries because most of their land mass is north of the Tropic of Cancer (latitude 23.27°N). The other 4 countries (Cambodia, Indonesia, Thailand, and Vietnam) are classified as tropical because they are located between the Tropics of Cancer and Capricorn (Table).

The 8 countries provided a total of 79 whole years (948 months) of data on outbreaks among poultry; 2013 data from Indonesia were not complete and therefore were not included in the analysis. Outbreaks were reported during 39% (365/948) of the study months. Most poultry H5N1 outbreaks (8,616/11,331, 76%) were reported during January–March. February had the highest total number of reported poultry H5N1 outbreaks, 3,118. Figure 2 provides the cumulative number of poultry H5N1 outbreaks reported each month by country and illustrates how these outbreaks clustered during January–March.

Poultry H5N1 Outbreaks and Human H5N1 Cases

During 2004–2013, the 8 study countries reported nearly all (625/645, 97%) human H5N1 cases (Table); cases were reported in 227 (24%) of the 948 study months. As seen with H5N1 outbreaks among poultry, half of human cases (313/625, 50%) occurred during January–March, and January had the highest number of reported H5N1 cases among humans ($\rho = 0.8$, $p = 0.004$) (Figures 2, 3). At least 1 human case of H5N1 infection occurred during 168 (45%) of the 374 months in which ≥1 outbreak in poultry occurred, compared with 59 (10%) of 574 months without outbreaks among poultry (relative risk = 5, $p<0.001$). After their initial identification, poultry H5N1 outbreaks and human H5N1 cases recurred concurrently during October–March in 24 (34%) of 70 study year equivalents.

Human H5N1 and Seasonal Influenza Activity

Seasonal influenza was epidemic in 267 (30%) of 888 months for which data were available. Human H5N1 cases occurred in 59 (22%) of these 267 months (Figure 4).

H5N1 Outbreaks in Poultry during Colder Temperatures

In each of the 8 countries, the number of poultry H5N1 outbreaks was highest during months with the lowest average ambient air temperature (Figure 2). In our regression model, colder ambient temperature was also ecologically associated with an increase in the number of poultry H5N1 outbreaks and human cases. For each decrease in temperature by 3.3°C, 1 additional poultry H5N1 outbreak was reported ($p = 0.004$), and for each decrease in temperature by 0.3°C, 1 additional H5N1 human case was reported ($p<0.001$). Precipitation was co-linear to temperature, however, and was dropped from the analysis.

Discussion

Our study reaffirms that, in Southeast Asia, H5N1 outbreaks among poultry and human H5N1 cases often

occur seasonally, during months when temperatures are relatively cool. Even when accounting for H5N1-endemic countries outside Southeast Asia, most (>50%) poultry H5N1 outbreaks and human H5N1 cases of H5N1 infection occurred during January–March. Human and animal health officials in affected countries should consider exploring the value of enhanced surveillance, flock biosecurity, and live bird market disinfection and/or rest days during periods when H5N1 viruses are typically detected (*22*–*24*).

Our analysis of 2004–2013 data from 8 countries also suggests that lower ambient temperatures are associated with H5N1 outbreaks among poultry, even though half of our data came from tropical countries, where annual temperature variations are often small. These results are similar to those described by Park and Glass, who observed poultry H5N1 outbreaks during 1997–2006 in Southeast Asia and China and concluded that these outbreaks most often occurred during colder months (*10*). Other studies have found similar associations (*5*,*25*,*26*). A decrease in temperature can

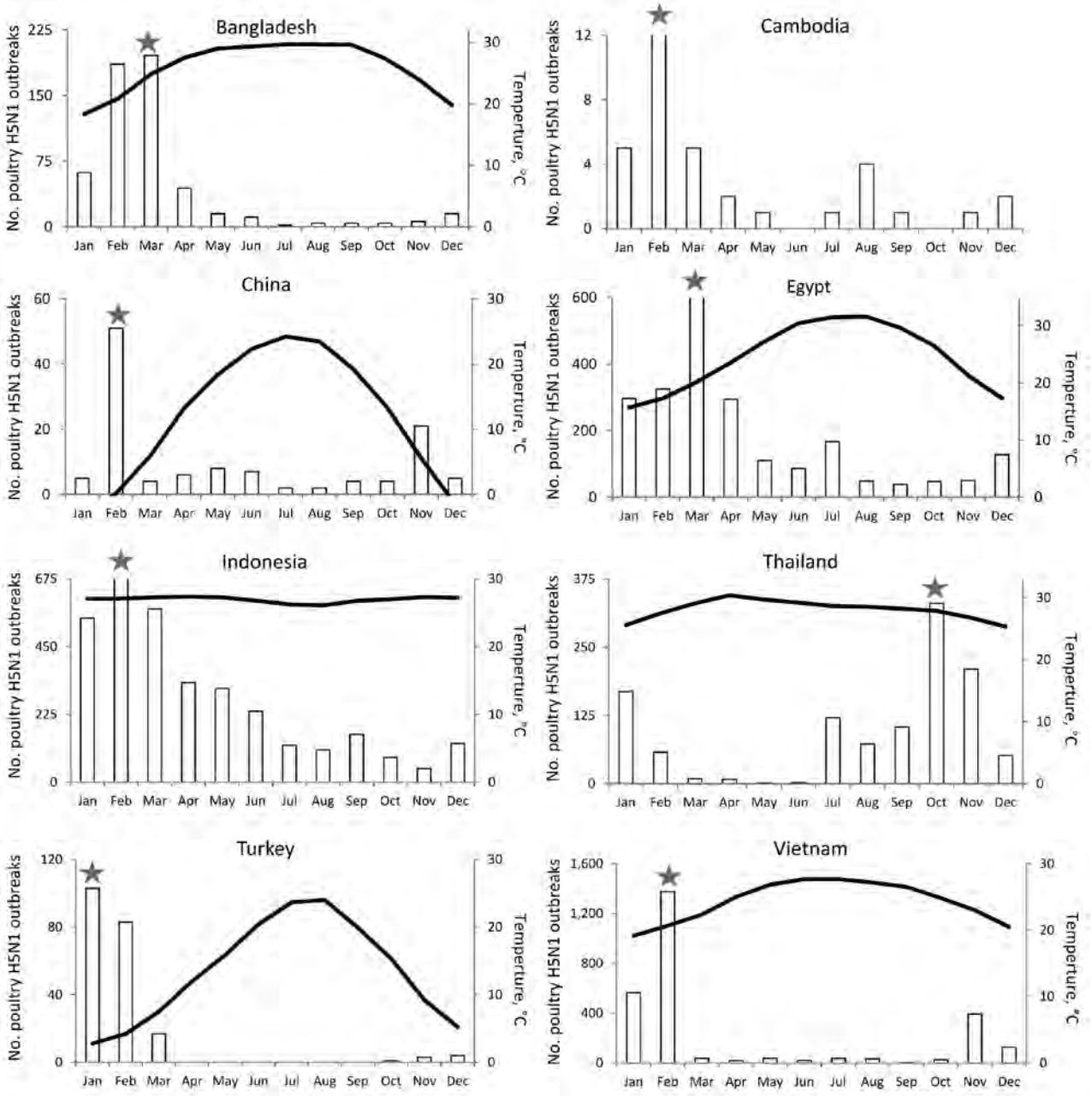

Figure 2. Outbreaks of highly pathogenic avian influenza A(H5N1) infection by month (white bars) and mean temperature (black lines) for the 8 countries that reported 90% of worldwide poultry H5N1 outbreaks during 2004–2013. Stars indicate month with highest average number of outbreaks for each country. Temperature data were not available for Cambodia.

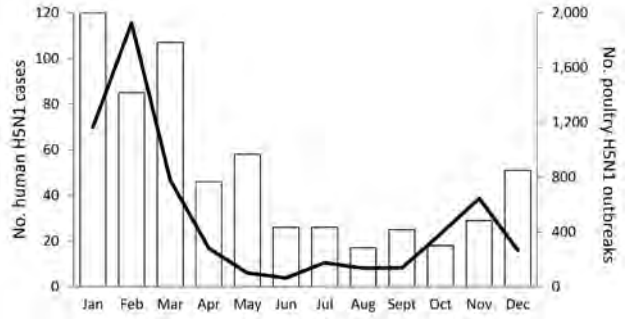

Figure 3. Monthly average number of highly pathogenic avian influenza A(H5N1) infection outbreaks among poultry (black line) and human H5N1 cases (white bars) for 8 study countries (Bangladesh, Cambodia, China, Egypt, Indonesia, Thailand, Turkey, and Vietnam) that reported 90% of all poultry H5N1 outbreaks and 97% of all human H5N1 cases during 2004–2013.

make poultry more susceptible to H5N1 because lower ambient temperature can decrease poultry immunity (*27–29*). Moreover, cold weather may enable prolonged viral survival in the secretions and feces of infected poultry, and anticipation of seasonal holidays (e.g., Chinese New Year) often results in increases in population density of domestic poultry and in trafficking of poultry (*27–34*).

Human H5N1 cases were almost 5 times more common in months during which poultry H5N1 outbreaks occurred. These findings reaffirm reports that human H5N1 virus infection is typically preceded by exposure to sick or dead poultry (*35*) and suggest that human and animal health officials in affected countries should explore the effectiveness of education and outreach efforts before and postexposure prophylaxis during anticipated H5N1 epidemic periods. These efforts could further enhance surveillance of H5N1 in poultry and lead to more prompt culling of poultry when H5N1 viruses are detected, thus helping to increase flock biosecurity and enable timely education and outreach efforts. For example, affected countries may want to evaluate the cost-effectiveness of targeted risk communication campaigns delivered before peak months of H5N1 infection to promote the avoidance of sick or dead poultry, the importance of hand washing, and the appropriate use of personal protective equipment among subpopulations that frequently are in contact with poultry. These efforts could help mitigate the transmission of H5N1 viruses among poultry and the spread of the virus to human populations (*22–24*).

Our data also suggest that one fifth of human H5N1 cases occurred in months during which seasonal influenza was epidemic. Concurrent H5N1 and human seasonal influenza activity provides opportunities for humans and other animals (e.g., swine) to become co-infected with these co-circulating viruses and for the viruses to reassort. Reassortment may generate novel influenza A virus strains with the ability to cause sustained human-to-human transmission.

Our findings support and demonstrate the feasibility of integrating human and animal surveillance for avian influenza and other zoonotic diseases. Combining the expertise from multiple surveillance systems can increase the detection rate of H5N1 cases in humans from 57% to 93% and of epizootics from 40% to 53% (*36*). This recommendation for an integrated H5N1 surveillance approach enables better prediction of disease risk and identification of outbreaks (*37*). Such approaches can assist in filling in gaps in human and animal surveillance systems (*36, 37*).

This study has several limitations. H5N1-endemic countries are not required to report immediate notifications. H5N1 reports also often are limited because of a lack of resources for surveillance, laboratory capacity to confirm H5N1, and, often, the political will to report outbreaks in poultry that may result in substantial economic losses (*38*). No formal case definition for a poultry H5N1 outbreak has been developed. During our study period, surveillance practices for human and poultry H5N1 and human seasonal influenza were not standardized; in fact, outbreaks among poultry might be more likely to be reported during periods when human H5N1 cases occurred. Further standardization of surveillance and reporting of influenza outbreaks in humans and animals might help clarify the epidemiology of these viruses (*19*).

Health authorities in H5N1 virus–affected countries should consider enhanced surveillance for H5N1 viruses among domestic poultry and humans in anticipation of cooler months, when H5N1 virus outbreaks are most likely to occur. Although year-round surveillance is important for the identification of novel strains of influenza, laboratorians should emphasize the identification, reporting, and sharing of nonsubtypable influenza A viruses during January–March. Public and animal health authorities may consider enhancing animal market surveillance during this time frame and encourage the use of personal protective equipment, disinfectant, and other resources for containment and mitigation in anticipation of increased H5N1 activity among poultry (*22–24*). These lessons may also be relevant to the management of avian influenza A(H7N9) virus outbreaks in China, which have occurred during similar times of the year.

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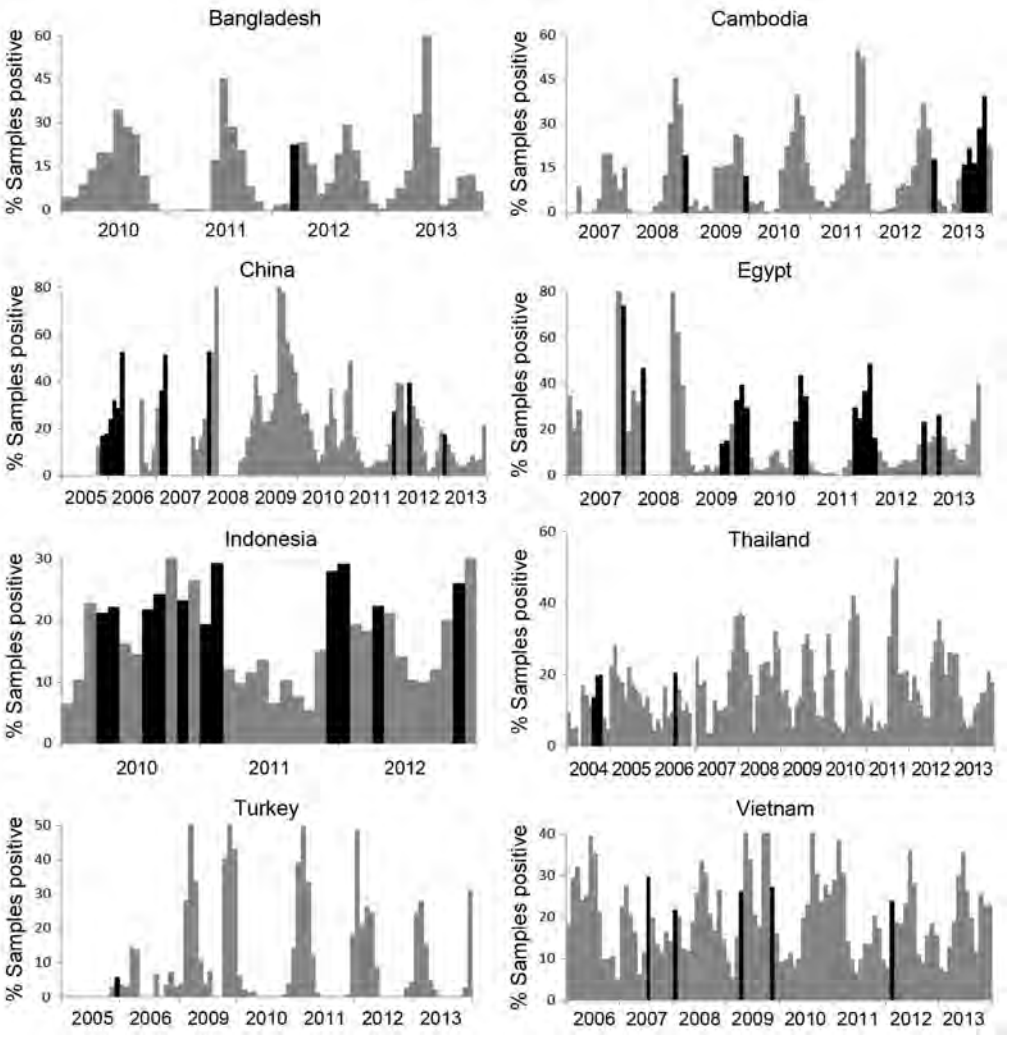

Figure 4. Human cases of highly pathogenic avian influenza A(H5N1) infection and seasonal influenza rates for 8 study countries, by month. Bars indicate the proportion of human respiratory samples that tested positive for seasonal influenza viruses; black bars indicate months during which seasonal influenza was epidemic and a human H5N1 case was reported. Years covered for each country are provided along *x*-axes.

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Quantifying Reporting Timeliness to Improve Outbreak Control

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The extent to which reporting delays should be reduced to gain substantial improvement in outbreak control is unclear. We developed a model to quantitatively assess reporting timeliness. Using reporting speed data for 6 infectious diseases in the notification system in the Netherlands, we calculated the proportion of infections produced by index and secondary cases until the index case is reported. We assumed interventions that immediately stop transmission. Reporting delays render useful only those interventions that stop transmission from index and secondary cases. We found that current reporting delays are adequate for hepatitis A and B control. However, reporting delays should be reduced by a few days to improve measles and mumps control, by at least 10 days to improve shigellosis control, and by at least 5 weeks to substantially improve pertussis control. Our method provides quantitative insight into the required reporting delay reductions needed to achieve outbreak control and other transmission prevention goals.

Timely reporting of infectious disease cases enables public health authorities (PHAs) to take effective action to prevent outbreaks by reducing disease transmission in a population. Therefore, many countries have notification systems for reporting infectious diseases to local PHAs. However, delays in the chain of reporting are inevitable. Figure 1 shows a schematic notification chain with its various delay links. The causes and durations of these links have diverse origins that must be individually analyzed to find possible ways of reducing them but only if reducing the total reporting delay (D_{OR} in Figure 1) proves worthwhile. Although any reduction of reporting delay provides individual benefit, aiming for overall reduction of the reporting delay makes sense at population level only if a given goal for improving outbreak control can be achieved. Therefore, the question arises as to whether PHAs should spend time, money, and effort to achieve effective improvement of the total reporting delay.

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Previous studies have found that for most diseases, the reporting delays are too long to prevent directly infected contacts from spreading the disease (*1*–*3*). Few studies have taken into account the full-time distribution of events in the reporting chain (*4*,*5*), and there has been no quantitative assessment of the effect of reporting delays on outbreak control. Moreover, assessing reporting timeliness by considering only time delay does not enable a comparison among different diseases because they generally develop over different timescales.

In this article, we show how to quantify reporting timeliness for outbreak control by calculating the proportion of infections expected to be caused by index cases and by their corresponding secondary cases (*6*) until the moment the index case is reported to a PHA. This approach enables not only quantitative assessment of the effect of reporting delay reduction for a particular disease but also comparison of reporting timeliness among different diseases. Our models take into account reporting delay distributions, generation (serial) interval distributions, and distributions of symptom-onset period. We used notification data for 6 infectious diseases reported to the Netherlands notification system to evaluate the current reporting timeliness and reporting delay reductions needed to substantially affect outbreak control. The effect of a reporting delay on new infections acquired from an index case (and subsequent secondary cases) indicates to public health officials the potential value of attempting to reduce the total reporting delay and the extent to which it may need to be done.

Methods

For evaluation of reporting timeliness, we selected 6 notifiable diseases that are transmitted person to person and for which sufficient data on total reporting delays (D_{OR}) are available in the notification system used in the Netherlands (OSIRIS): hepatitis A, hepatitis B, measles, mumps, pertussis, and shigellosis. For 5 of those diseases, we obtained data for day of symptom onset and day of reporting to the PHA for all cases reported from July 2003 through December 2011. The other disease, mumps, was notifiable in the Netherlands until 1999, when it was dropped from the notifiable list because of a decreased number of cases. However, after a resurgence in the number of cases in 2008, mumps was reintroduced as a notifiable disease in OSIRIS in 2009. Therefore, no mumps data were available during

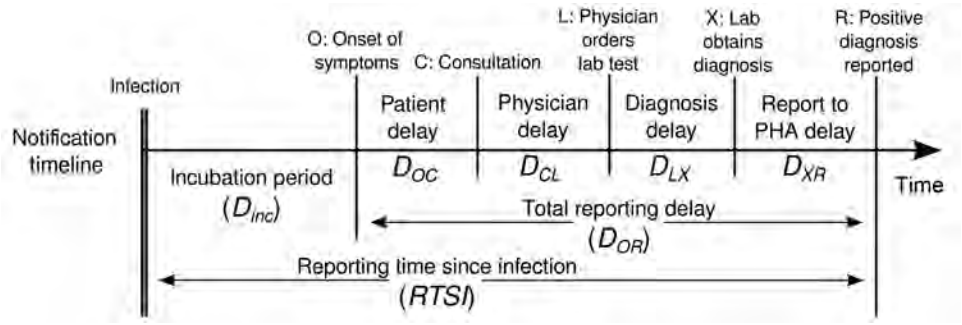

Figure 1. Timeline for chain of disease reporting, the Netherlands. Lab, laboratory; PHA, public health authority.

1999–2008. We included mumps in our analysis because of its high incidence even though control measures are limited, similar to the situation with pertussis. To model the reporting delay for each disease, we fitted analytical log-normal distributions to the OSIRIS data. We also used log-normal distributions that fit serial interval and time-to-symptom onset ranges found in the literature (Table 1).

The course of infection for each disease has its own characteristic time scale (latent, infectious, and symptomatic periods). Thus, a 1-week delay might have a substantial effect on control of a slowly progressing disease such as hepatitis A but not on a rapidly progressing disease such as shigellosis. Moreover, reporting itself also has its own time scale because of various factors behind each link in the reporting chain. Therefore, for timeliness of case reporting to be assessed and compared for various diseases, timeliness needs to be evaluated in terms of the number of infections that could not be prevented because of the delay, rather than in terms of the actual time taken to report cases.

When a case is reported, regional PHAs implement mostly case-based interventions. These interventions are intended to prevent transmission from the reported case and from secondary cases that may have been acquired from the index case. Secondary cases are identified by contact tracing. For this reason, for each disease we first calculated the proportion of expected infections produced by an index case (PIR1) until the moment the index case in question is reported to the local PHA. We then calculated the proportion of expected infections produced by each secondary case produced by a reported index case (PIR2) until the moment the index case in question is reported to the local PHA. Throughout this study we refer to an index case as any case that is reported because of a positive diagnosis and a case that has not yet been traced as a secondary case when reported (i.e., all primary cases that may result in clusters). For every calculation, we considered the hypothetical intervention in which contact tracing and stopping of transmission occur instantly when the index case is reported. Such a rapid response is not realistic, but the estimate provides an upper limit for outbreak control potential as determined by reporting speed. The calculations were performed by using scripts written in Python programming language (https://www.python.org). Below is an introductory explanation of our calculations; further details and explicit formulas are provided in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/2/13-0504-Techapp1.pdf).

Calculation of PIR1, a 1-Generation–based Response

The generation interval is the time elapsed from the moment an infector acquires a pathogen until he/she infects another host. The distribution of the generation interval time then indicates the average infective profile since an index case acquired the pathogen (*15*). Figure 2, panel A, shows distribution of an index case generation interval: initially a period when no infections take place (latent period); then as time passes, the profile rises (beginning of infectious period) to a peak (most infections occur at this moment) and later declines, leading to the end of the infectious period. We considered the reporting time elapsed since infection (RTSI) to be the addition of the symptom onset time

Table 1. Parameters for reporting delay models, by disease*

Disease	Serial interval distribution, median days (SD)	Symptom onset distribution, median days (SD)	Reporting delay distribution, median days (SD)	Reproduction number, R (range)†	References
Hepatitis A	27.5 (4)	28 (9)	8.6 (11.9)	3.33 (3–4)	(*7*–*10*)
Hepatitis B	47.5 (20)	80 (35)	14.7 (24.3)	1.75 (1–2.5)	(*7*–*9*,*11*)
Measles	11.6 (2.4)	11.5 (2.5)	9.0 (12)	8 (8–30)	(*7*–*10*,*12*)
Mumps	19.1 (5.4)	19.5 (2.3)	9.0 (13.8)	5.5 (4–7)	(*7*–*10*)
Pertussis	16 (13)	9 (2.5)	40.8 (24.4)	5.5 (5–6.5)	(*7*–*10*,*13*,*14*)
Shigellosis‡	5 (3.5)	2.5 (1.5)	14.6 (13.8)	3.5 (2–5)	(*7*–*9*)

*All distributions are fitted to log-normal distributions with medians and standard deviations as indicated. Reporting delay distribution of pertussis is an exception, which is fitted to a gamma distribution.
†The reproduction numbers are those used for outbreak control calculations, and the ranges in brackets are those found in the literature.
‡For shigellosis, an average transmission period (serial interval distribution) of 1 wk (median 5 d) was assumed, although in practice shedding continues after that.

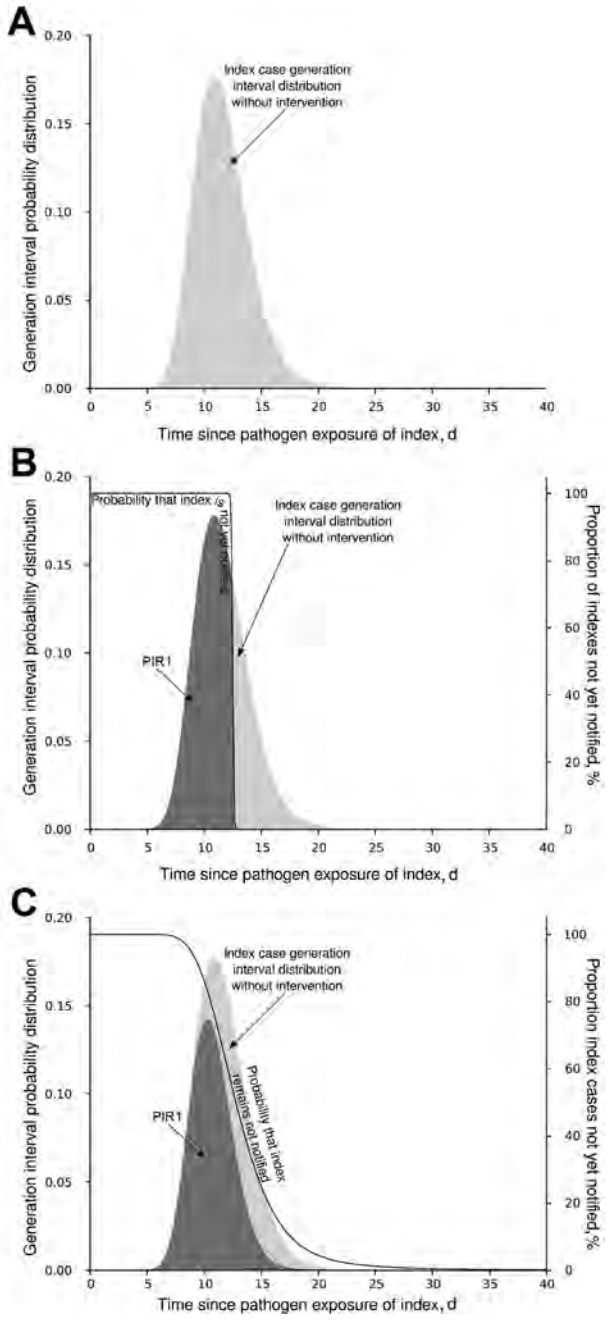

Figure 2. Schematic modification of PIR1. A) Generation interval distribution of an index case as function of time since the index case acquired the pathogen. Without notification and intervention, the proportion of infections expected by the index case is 1, the light gray area under the curve. B) How the generation interval distribution is modified, assuming that all index cases are notified and stopped exactly 13 days after exposure to the pathogen. C) How the average generation interval is modified when index cases are notified and stopped according to a time distribution. Dark gray shading indicates the PIR1 value for each situation. The black line indicates the proportion of index cases not yet notified (right y-axis), equivalent to the probability of an index case not yet being notified in each situation. PIR1, expected proportion of cases caused by index case at notification.

(D_{INC}) and the reporting delay (D_{OR}) (Figure 1). Figure 2, panel B, shows PIR1 in the extreme case that RTSI is fixed at 13 days for every infector, meaning that the probability of not being reported before RTSI is 1 and after RTSI is 0. The proportion of expected infections produced by an index case (PIR1) until reporting time is then provided by the area under the generation interval curve weighted by the probability of not yet being reported, equivalent to the percentage of nonreported cases. However, times for symptom onset and reporting delays vary from person to person, and RTSI then becomes a distribution. This distribution smooths out the step-like probability of any infector not being reported at a given time (Figure 2, panel C), which consequently smooths out how the generation interval must be weighted for an appropriate PIR1 calculation.

Calculation of PIR2, a 2-Generation–based Response

The calculation of the proportion of expected infections produced by secondary cases at the time when their corresponding index case is reported (PIR2) is conducted in the same way as that of PIR1, but a 2-generation interval is used instead of a standard generation interval. A 2-generation interval is constructed by subsequently adding 2 standard generation intervals. The 2-generation interval distribution indicates the average second-generation infective profile as time passes since the index case acquired the pathogen (Figure 3, panel A). PIR2 is then provided by the area under the 2-generation interval curve weighted by the probability of the index case not yet being reported (Figure 3, panel B).

At an early stage, an outbreak is controlled (i.e., prevalence begins to decline) by implementing a case-based intervention that can stop transmission early enough so that the number of cases produced per infector is <1 (*6*,*16*,*17*). The number of cases produced per index case is calculated by multiplying PIR1 times the reproduction number (R) of the disease in question. The number of cases produced per secondary case is PIR2 multiplied by the reproduction number. Hence, for each disease, we assumed that the conditions for outbreak control are PIR1<1/R and PIR2<1/R. In addition, given that PIR2 involves 2 generations, we considered the alternative (more restrictive) outbreak control condition PIR2<1/R^2. To evaluate the status of the current reporting timeliness of the 6 diseases, we compared our PIR1 and PIR2 results with the outbreak control conditions.

To assess the potential for improvement by reducing current reporting delays for each disease, we studied how much various reporting delays influence PIR1 and PIR2 and calculated the reporting delay reductions needed to reach outbreak control conditions. PIR1 and PIR2 were highly dependent on the reporting delay median but not so regarding standard deviations within the range matching actual reporting delay distributions (online Technical Appendix

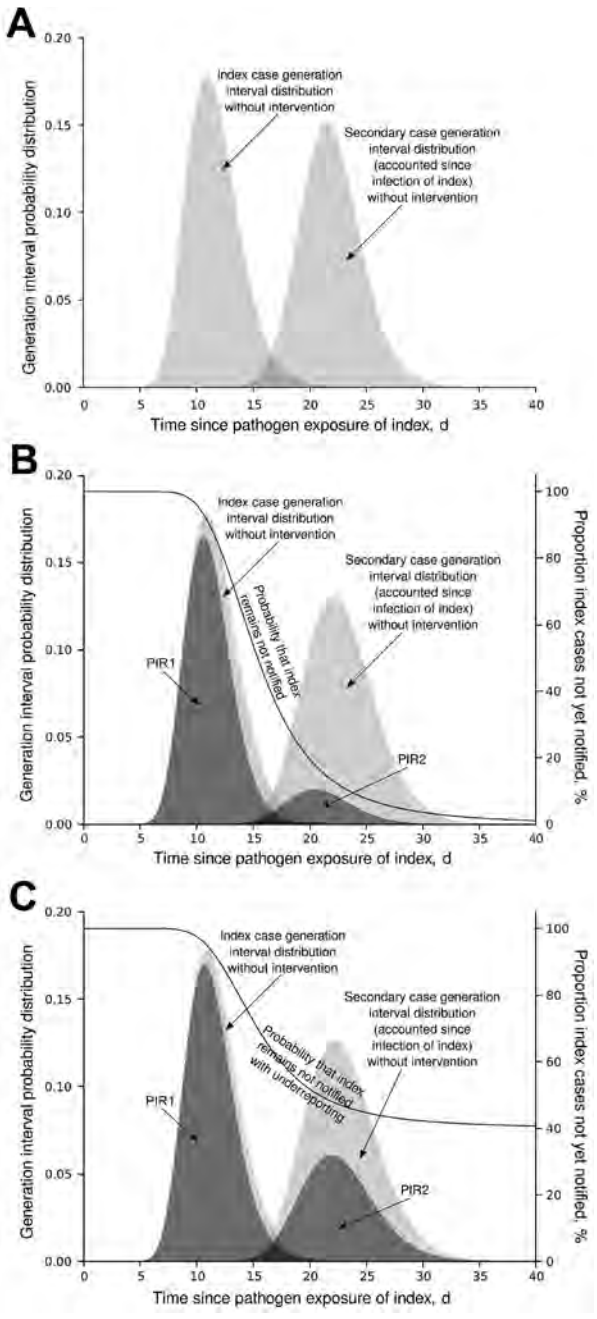

Figure 3. Schematic modification of PIR2. A) Generation interval time distributions of index and secondary cases, from the moment of exposure of the notified index case. PIR2 is represented by the area under the second generation interval distribution, which is 1 in the absence of notification/intervention. B) PIR1 and PIR2 values when index cases are notified and stopped together with their secondary cases, according to a time distribution. C) How PIR values in panel B are modified by 40% underreporting. Dark gray shading indicates PIR1 and PRI2 values. The black line indicates the proportion of index cases not yet notified (right y-axis), equivalent to the probability of an index case not yet being notified in each situation. PIR1, expected proportion of cases caused by index case at notification; PIR2, expected proportion of new infections caused by secondary cases before index case is notified.

Figure). Therefore, for simplicity, we present our reporting delay analysis results as medians of 1–60 days under the assumption that standard deviations are equal to the medians**.** For each disease, we evaluated the ratio at which PIR1 and PIR2 are reduced after the reporting delay median is reduced by 1 day, as extracted from the OSIRIS database. These reduction ratios enabled us to determine those diseases for which reduction of reporting delays would most prevent further transmission.

Underreporting

Our calculations of PIR1 and PIR2 were made with the assumption of 100% reporting compliance. However, a proportion of cases are not reported (and might include asymptomatic cases). From an outbreak control point of view, underreporting can be tackled by assuming that there are only reported cases, each producing an increased average number of infections to account for the contribution to disease transmission from the cases that are not reported (Figure 3, panel C). PIR1 and PIR2 are modified as follows: PIR (underreported) = PIR × (1 – proportion.underreported) + proportion.underreported. Therefore, there is a maximum limit for underreporting beyond which it is not possible to satisfy the outbreak control condition R × PIR (underreported) <1. We calculated this limit for the studied diseases by assuming instantaneous reporting at the day of symptom onset. Although this assumption is not realistic, it provides an upper limit estimate for underreporting if outbreak control is desired.

Vaccination Coverage

Many diseases are preventable by vaccination; among the 13 diseases in the National Immunization Program vaccination schedule for the Netherlands are hepatitis B, measles, mumps, and pertussis. Consequently, part of the population might be protected by vaccine-induced immunity. When considering that individually targeted interventions are implemented effectively, the minimum vaccination coverage needed to achieve herd immunity is reduced. We calculated this reduction for the 6 studied diseases by first considering the current reporting delays and the outbreak control condition $[R(1 - \text{coverage})]^2 \times \text{PIR2} < 1$. From this condition we derived the reduced minimum vaccination coverage needed for outbreak control and compared it with the standard vaccination coverage needed for achieving herd immunity (coverage>1 – 1/R).

Model Parameters

We fitted log-normal distributions to frequency distribution of reporting delays as extracted from OSIRIS. We performed fitting with a Kolmogorov-Smirnov minimization and a 0.05 significance level by using the program Mathematica version 8.0 (http://www.wolfram.com/mathematica/).

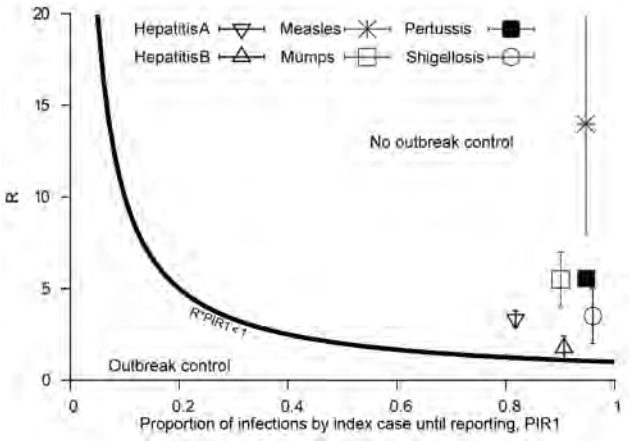

Figure 4. Timeliness of current reporting speed in the Netherlands, evaluated considering interventions applied to reported index cases only. The outbreak control condition is R × PIR1<1. Diseases that lie in the outbreak control areas are those for which reporting speed is timely enough to enable outbreak control. PIR1, expected proportion of cases caused by index case at notification; R, reproduction number.

Table 1 shows the parameters we found and used in our models for each disease.

Because generation intervals are difficult to observe, we used the serial interval as a proxy for the generation interval (*18*) and assumed equivalence. The serial interval is the time between symptom onset of an index case and symptom onset of a secondary case. For each disease we extracted information on incubation period distribution, serial interval, and reproduction number from the published literature (Table 1). Most data on incubation periods and serial intervals in the literature are provided as a range with a relevant value (average, median, or mode). Therefore, we constructed the distributions by finding parameters for which a log-normal distribution would present the relevant value found in the literature, and the 2.5th and 97.5th percentiles would correspond to the ranges found in the literature. Log-normal distributions are easy to handle, and there is evidence favoring them as incubation period distributions (*19*).

Results

Current Reporting Timeliness

Current reporting timeliness in the Netherlands is shown in Figure 4; calculations are based on data from the Netherlands and consider interventions applied only to the reported index case. For most diseases, the expected proportion of infections produced until reporting is >90%. The expected proportion of infections is lower for hepatitis only; however, this proportion is still high at >80%. Therefore, if an index case is instantly removed as a source at the moment of reporting, in general ≤10% secondary infections are prevented, which renders such an intervention rather ineffective. Even fewer infections can be prevented if underreporting is considered. All diseases shown in Figure 4 lie above outbreak control condition.

When interventions are also applied to secondary cases produced by a reported index case, the interventions become more effective. The expected proportion of infections produced by secondary cases, PIR2, for the 6 diseases is shown in Figure 5. Hepatitis A lies close to the lower outbreak control condition, indicating that its current reporting speed should be timely enough to keep it under control. The same indication applies to hepatitis B, which also lies below the upper outbreak control limit, despite its intermediate PIR2 values. PIR2 for measles is intermediate, which places the disease far outside the area in which control is possible. PIR2 is low to intermediate for mumps, but the reproduction number for mumps is smaller than that for measles, which places the disease close to the upper outbreak control condition. Pertussis and shigellosis remain in the region of high PIR2 values, meaning that secondary cases may have already produced most infections at the moment that the index case is reported, thereby limiting the effectiveness of outbreak control by means of contact tracing.

Room for Improving Reporting Timeliness

Interventions applied only to index cases are rather inefficient, even with the swiftest reporting. Figure 6 shows that even when a case is (unrealistically) reported on the same day of symptom onset (median = 0), PIR2 values are above their respective outbreak control limit for 5 of the 6 studied diseases. This finding is because of the proportion of expected secondary infections an index case produces while asymptomatic (*6*). The exception is shigellosis, but for this disease already a median reporting delay of 1 day would be too late for implementing outbreak control. In general, only short reporting delays of ≈3 days would enable substantial reduction of PIR1 for the 6 diseases. Moreover, with current reporting delays, the largest PIR1 reduction ratio achieved by reducing current delays by 1 day is 2.6% (for hepatitis A) (Table 2).

However, for some diseases, the efficiency of applying interventions also to traced contacts can be substantially increased by reducing reporting delays. Relevant results are summarized in Table 2. Figure 7 shows that for sufficiently short reporting delay medians, the upper outbreak control condition could eventually be satisfied for all 6 diseases. If the median reporting delay for hepatitis A were 8 days, the lower, more restrictive, outbreak control condition could be satisfied. Outbreak control for measles and mumps would need reporting delay medians of 2 and 3 days, respectively. For hepatitis B and shigellosis, reporting would need to be almost instantaneous (1-day delay); for pertussis, the lower

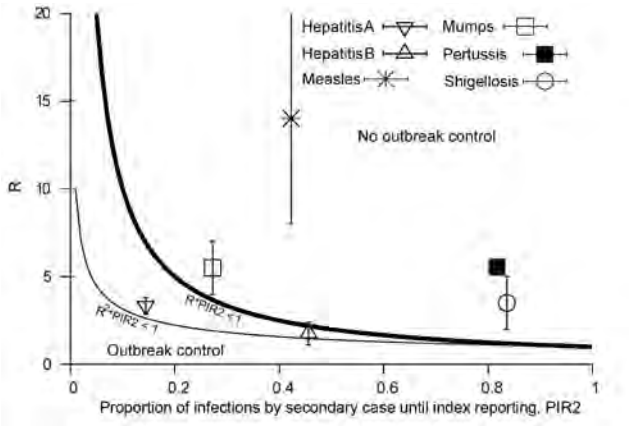

Figure 5. Timeliness of current reporting speed in the Netherlands, evaluated considering interventions applied for reported index cases and their secondary cases. The lower outbreak control condition is $R^2 \times PIR2<1$, assuming index cases are reported too late to stop any secondary infection (i.e., PIR1 = 1 always). The upper outbreak control condition $R \times PIR2<1$, which is the most relaxed condition, assumes an extreme situation that index cases have not caused more infections than secondary cases (PIR1 = PIR2). In practice, the outbreak control condition lies in between these 2 condition. Diseases that lie in the outbreak control areas are those for which reporting speed is timely enough to enable outbreak control. PIR1, expected proportion of cases caused by index case at notification; PIR2, expected proportion of new infections caused by secondary cases before index case is notified; R, reproduction number.

outbreak control condition could not be satisfied even if reported the same day as symptom onset. The PIR2 reduction ratio is high for hepatitis A, measles, and mumps, indicating that substantial improvement can be achieved with reporting delay reductions of 1 (or a few) days. However, little improvement is expected with a small reduction of reporting delays for hepatitis B, pertussis, and shigellosis. Table 2 shows that with underreporting >30%, outbreak control for all 6 diseases is not possible. Table 2 also shows that only for hepatitis A and hepatitis B would the immunization coverage needed to achieve outbreak control be substantially reduced, because of individually targeted interventions with the current reporting speed.

Discussion

Public health agencies are responsible for organizing a swift course of action from disease onset to notification and intervention. The general assumption is that the shorter the delay, the better the control response. We quantitatively assessed the potential of individual-based interventions by PHAs for minimizing or preventing outbreaks by calculating the expected proportion of infections caused by index (PIR1) and secondary (PIR2) cases until reporting of the index case. For hepatitis A, measles, mumps, pertussis, and shigellosis, PIR1 was >90% (Figure 3), but for hepatitis B, PIR1 was 82%. This finding indicates that interventions aimed only at notified index cases are always too late to substantially prevent future disease transmission. Therefore, effective control requires contact tracing and stopping transmission from contacts. Even if reporting delays were reduced to a couple of days, interventions targeting index cases only are not enough to achieve outbreak control conditions.

The expected proportion of infections caused by secondary cases, PIR2, differs substantially among different diseases. Current reporting delays for hepatitis A and hepatitis B lead to PIR2 values that are within outbreak control limits (Figure 5), probably because of the long incubation period for each disease. Although incubation periods for pertussis and shigellosis differ greatly, reporting for these diseases is far from meeting outbreak control conditions (Figure 5), probably resulting partly from patient and physician reporting delays because of nonspecific symptoms. Measles and mumps appear in the middle of Figure 5, despite their relatively short incubation periods, probably because they produce specific symptoms. For measles and mumps, a combined effort to lower their reproduction number and shorten notification delay might bring their reporting within outbreak control conditions. Lower reproduction numbers move diseases to a lower position in Figures 4 and 5. This lowering can be achieved by interventions at population or group levels (e.g., vaccination or hygiene/behavior changes). Areas with high baseline vaccination ratios against measles or mumps (boosters included) are closer to meeting outbreak control conditions than those with low

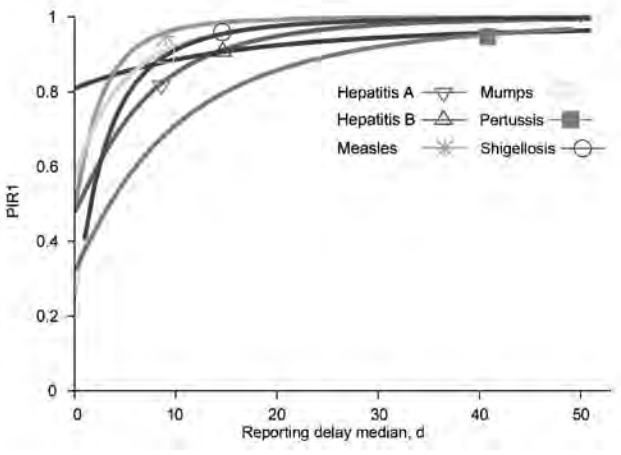

Figure 6. PIR1, depending on reporting delay median for the indicated diseases and assuming standard deviation equal to median value. Thick lines show reporting delay medians for which there is no outbreak control. Thin dashed lines would show reporting delay medians that bring diseases within the outbreak condition ($R \times PIR1<1$), but they are not present because even with extremely short delays it is not possible to fulfill the condition with the studied diseases (except shigellosis). Symbols indicate PIR1 evaluated with current reporting delay data. PIR1, expected proportion of cases caused by index case at notification; R, reproduction number.

Table 2. Effects of reducing reporting delays, by disease*

Effect	Hepatitis A	Hepatitis B	Measles	Mumps	Pertussis	Shigellosis
Current PIR1 (PIR1 at symptom onset)	0.818 (0.480)	0.907 (0.810)	0.947 (0.495)	0.901 (0.574)	0.948 (0.322)	0.960 (0.230)
Current PIR2 (PIR2 at symptom onset)	0.145 (0.019)	0.456 (0.320)	0.423 (0.006)	0.273 (0.005)	0.817 (0.067)	0.836 (0.056)
PIR2 reduction ratio by reducing delay in 1 d	9.7%	1.5%	12.6%	13.5%	0.7%	2.4%
Reporting delay median needed for PIR2 = 1/R	17 d	42 d	5 d	8 d	4.5 d	3 d
Reporting delay median needed for PIR2 = $1/R^2$	8 d	1 d	2 d	3 d	Not possible	1 d
Underreporting beyond which outbreak control is not possible	29%	1.5%	12%	18%	12%	25%
Reduction of vaccination coverage for herd immunity	70%	64%	8%	20%	2%	4%

*PIR values at symptom onset show a theoretical minimum, achievable by stopping transmission instantly at symptom onset. PIR1, proportion of expected infections produced by an index case; PIR2, proportion of expected infections produced by each secondary case produced by a reported index case.

baseline vaccination ratios and need less drastic delay reductions to achieve the same effect when interventions are applied to secondary cases.

Whether a PHA response to a reported case is considered timely depends on the goal set for the intervention and the balance between the benefit and efforts spent on reducing reporting delays. PIR2 decreases substantially with a reporting delay reduction of a few days for hepatitis A, measles, and mumps. This finding suggests that efforts to reduce reporting delays for these diseases would be worthwhile because doing so would increase the effectiveness of interventions applied to secondary cases. However, using resources to reduce reporting delays for hepatitis B, pertussis, and shigellosis would not be worthwhile for outbreak control purposes because a substantial increase of prevented infections could be achieved only with extreme reporting delay reductions (pertussis and shigellosis) or none at all (hepatitis B).

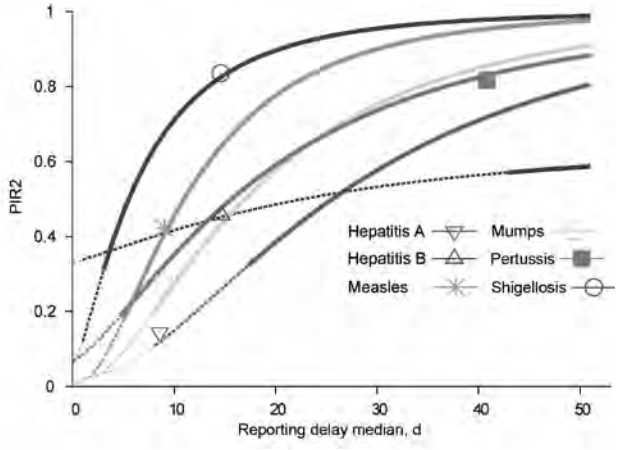

Figure 7. Expected proportion of infections caused by secondary cases before reporting of their index case, depending on reporting delay median for the indicated diseases and assuming standard deviation equal to median value. Thick lines show reporting delay medians for which there is no outbreak control. Intermediate-width dashed lines show reporting delay medians that bring diseases within the upper outbreak condition (R × PIR2<1). Thin dashed lines show reporting delay medians bringing diseases under the lower outbreak control condition (R^2 × PIR2<1). Symbols indicate PIR2 evaluated with current reporting delay data. HepA, hepatitis A; hepB, hepatitis B; PIR2, expected proportion of new infections caused by secondary cases before index case is notified; R, reproduction number.

In the context of transmission of infection, calculations of PIR1 and PIR2 provide an objective measure for the timeliness of reporting and interventions. We focused on outbreak control as a goal (reducing reproduction number to <1), but the method we described can also be used to assess reporting delays with another goal in mind. For example, for an extremely serious disease, the goal might be to reduce PIR1 and PIR2 to the smallest acceptable limit.

Maximum limits of underreporting that would allow for any possibility of outbreak control are rather small (Table 2). Therefore, in addition to being timely, reporting must also be very complete.

The median is a robust characteristic of a dataset because it is not influenced largely by outliers. Therefore, medians have been used in many studies to compare data on factors such as latent periods, serial intervals, and notification delays but without taking into account the shape of the distributions (*1*–*3*). It is reassuring that we found that calculations of PIR1 and PIR2 depend mainly on medians and not on the standard deviations of reporting delay distributions.

We have quantitatively assessed the outbreak control potential of PHA responses on the basis of the timeliness of the current reporting delays of hepatitis A, hepatitis B, measles, mumps, pertussis, and shigellosis in the Netherlands. We used the expected proportion of infections caused by index and secondary cases until reported to the local PHA (PIR1 and PIR2). These disease-specific quantities provide a powerful tool for setting goals for reporting speeds, not only for outbreak control but also for evaluation of individual-based interventions with other aims, such as partially reducing infections or completely stopping transmission.

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Tickborne Relapsing Fever, Bitterroot Valley, Montana, USA

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In July 2013, a resident of the Bitterroot Valley in western Montana, USA, contracted tickborne relapsing fever caused by an infection with the spirochete *Borrelia hermsii*. The patient's travel history and activities before onset of illness indicated a possible exposure on his residential property on the eastern side of the valley. An onsite investigation of the potential exposure site found the vector, *Ornithodoros hermsi* ticks, and 1 chipmunk infected with spirochetes, which on the basis of multilocus sequence typing were identical to the spirochete isolated from the patient. Field studies in other locations found additional serologic evidence and an infected tick that demonstrated a wider distribution of spirochetes circulating among the small mammal populations. Our study demonstrates that this area of Montana represents a previously unrecognized focus of relapsing fever and poses a risk for persons of acquiring this tickborne disease.

Seminal research on tickborne diseases of humans in North America began more than a century ago with the discovery in 1906 that an illness locally called black measles, which affected persons in the Bitterroot Valley of western Montana, USA, resulted from the bite of a bacteria-infected Rocky Mountain wood tick (*1*,*2*). What soon followed was the establishment of a multidisciplinary public health program to control this newly identified disease, now called Rocky Mountain spotted fever, which was caused by *Rickettsia rickettsii*, and a search was conducted for other diseases in nature that resulted from the bite of pathogen-infected ticks. These programs were based at a newly funded state laboratory in Hamilton in the Bitterroot Valley, a facility that was soon incorporated into the US Public Health Service and is now the Rocky Mountain Laboratories (RML) of the National Institute of Allergy and Infectious Diseases.

One of the many diseases studied at the RML since the early 1930s has been tickborne relapsing fever (RML, unpub. data) (*3*,*4*). In North America, this zoonosis is associated with 3 species of spirochetes, but most human cases are caused by *Borrelia hermsii*, which is found in scattered foci in the western United States and southern British Columbia, Canada (*5*,*6*). The specific vector of this spirochete is the *Ornithodoros hermsi* tick (*7*), which is found in higher-elevation coniferous forests where its preferred rodent hosts, primarily squirrels and chipmunks, are also found (*6*). In spite of the many decades of intensive research on ticks and tickborne diseases in the Bitterroot Valley, the tick *O. hermsi*, the spirochete *B. hermsii*, or an autochthonous human case of relapsing fever has not been observed in this region of Montana, until now. We report a case of tickborne relapsing fever in a person in this region.

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Case-Patient

The patient was a previously healthy 55-year-old man who sought care in July 2013 after a week of fevers. He had traveled widely over the previous months, including trips to Antarctica, New Zealand, Spain, Italy, and eastern Washington State, before returning to his home in the Bitterroot Valley of Montana. A week before his symptoms developed, he had moved part of a woodpile and noted rodent feces among the cut logs. He came to the emergency department of a local hospital because of fever, chills, night sweats, fatigue, nausea, vomiting, diarrhea, and malaise.

At initial evaluation, he had a temperature of 37.3°C; other vital signs were within reference ranges. Initial laboratory values included mildly increased levels of bilirubin, alkaline phosphatase, and aspartate aminotransferase, and mild transaminitis with an increased level of alanine aminotransferase (Table 1). A complete blood count showed thrombocytopenia (platelet count 51,000/mL). Hemoglobin level and leukocyte count were within reference ranges. He was given a presumptive diagnosis of an acute viral infection and discharged from the emergency department.

He returned 2 days later with confusion, tachycardia, hypoxemia, and a measurable fever (temperature 38.7°C). A physical examination identified a bilateral conjunctival suffusion, more notable in the right eye; cervical lymphadenopathy, tachypnea, tachycardia, splenomegaly, and major confusion. Additional laboratory tests showed a leukocyte count of 9,400 cells/mm^3 with 23% bands and a further reduced platelet count of 29,000/mL. Levels of bilirubin, alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, serum lactate with lactic acidosis, procalcitonin, and total creatine kinase were all highly

Table 1. Laboratory test results for serum from a 55-year-old man with tickborne relapsing fever on 2 dates compared with reference values, Montana, USA, 2013

	Blood components examined*								
Characteristic	Platelets/mL	Bilirubin, mg/dL	ALP, IU/L	AST, U/L	ALT, U/L	Serum lactate, mmol/L	PCT, μg/L	LDH, U/L	CK, U/L
Sample date									
Jul 20	51,000	1.7	234	89	97	ND	ND	ND	ND
Jul 22	29,000	6.8	414	215	144	3.9	7.99	495	602
Reference range or value	150–450,000	0.2–1.9	45–150	14–59	10–55	0.5–2.2	<0.05	300–600	20–200

*ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PCT, procalcitonin; LDH, lactate dehydrogenase; CK, creatine kinase; ND, not determined.

increased above reference values (Table 1). Urinalysis showed a 2+ protein level, and a chest radiograph showed diffuse bilateral pulmonary infiltrates.

He was admitted to the hospital and given intravenous ceftriaxone and oral doxycycline pending further examination. A peripheral smear was prepared, which showed thrombocytopenia and multiple extracellular spirochetes. A coded diagnostic specimen of the patient's whole blood was sent to the RML for identification of the spirochetes. His hospital course was notable for acute respiratory failure caused by acute respiratory distress syndrome, metabolic encephalopathy, and uveitis of the right eye. His sepsis and acute respiratory distress syndrome resolved, and his condition rapidly improved after treatment with ceftriaxone and doxycycline. On hospital day 5, he was discharged and given a 14-day course of oral doxycycline.

He was seen as an outpatient and showed resolution of abnormalities and symptoms, except for decreased visual acuity of the right eye that ultimately required a corrective lens. Results of serologic tests performed at a commercial diagnostic laboratory were negative for antibodies against *Coxiella burnetii*, *Brucella* spp., *Francisella tularensis*, *Leptospira interrogans*, *Treponema pallidum*, hantavirus (Sin Nombre virus), Colorado tick fever virus, cytomegalovirus, Epstein-Barr virus, and hepatitis A, B, and C viruses. An IgM test result for Lyme disease was equivocal but was probably cross-reactive because of the *B. hermsii* infection. A convalescent-phase serum sample obtained 6 weeks after onset of symptoms was sent to the Centers for Disease Control and Prevention (Fort Collins, CO, USA). This sample was positive for *B. hermsii* by enzyme immunoassay and Western blotting.

Materials and Methods

The coded diagnostic sample of the patient's blood obtained on July 22, 2013 was received at RML 3 days later. Thin blood smears were prepared on microscope slides, fixed with 100% methanol, and examined by using indirect fluorescent antibody staining with monoclonal antibodies H9724 (*8*) and H9826 (*9*) to identify spirochetes.

Motile spirochetes were observed in the blood by darkfield microscopy, and 100 μL of sample was inoculated intraperitoneally into 1 laboratory mouse (*Mus musculus*) to amplify and isolate spirochetes as described (*10*). Spirochetes in mouse blood were isolated in Barbour-Stoenner-Kelly x medium (*11*,*12*) containing 12% rabbit serum.

Multilocus sequence typing (MLST) of spirochetes was performed with purified genomic DNA samples extracted from bacterial cultures. Seven genetic loci were examined: partial sequences for the 16S rRNA (1,273 bp) and intergenic spacer (663 bp); and complete sequences for *flaB* (1,002 bp), *gyrB* (1,902 bp), *glpQ* (1,020 bp), *fhbA* (555 bp), and *vtp* (627 bp). Approximately 7,042–7,111 bp were determined for each isolate on which to base species identifications. The primers, PCR conditions, and methods for DNA sequencing have been described (*13*–*15*).

Given the patient's date of onset of illness, travel history, and reported incubation periods for the infection (average 7 days, range 4–18 days) (*16*), we concluded that he was possibly infected on his own property. Therefore, we conducted a short on-site investigation to search for supporting evidence that he could have been infected there. Live traps (Tomahawk Live Traps, Hazelhurst, WI, USA, and H.B. Sherman Live Traps Inc., Tallahassee, FL, USA) were set on the property on the evening of August 6 to the morning of August 8, 2013. The animals captured were anesthetized with isoflurane, blood was collected from the tail vein, standard morphometric data were collected for each animal, and they were identified to species on the basis of published descriptions of Montana wildlife (*17*). A skin punch biopsy was taken from 1 external ear for DNA extraction and determination of the mitochondrial *cytB* gene sequence to confirm the identifications (*18*), and the animals were released at the location of capture.

This work was conducted with approval of the RML Animal Care and Use Committee (protocol #2012–029 and #2013–035), the Montana Department of Fish, Wildlife and Parks (permit #2013–104) and the Bitterroot National Forest (permit #BIT201310). Work with animals was conducted in accordance with the institution's guidelines for animal husbandry, and followed the guidelines and basic principles in the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

Blood samples from the wild rodents were examined for spirochetes as wet mounts with a dark-field microscope, and as fixed, Giemsa-stained thin blood smears with a bright-field microscope. Serum samples (1:100 dilution) were tested by immunoblot with a *B. hermsii* whole-cell lysate and purified recombinant glycerophosphodiester phosphodiesterase (rGlpQ) as described (*19*,*20*).

CO_2 traps baited with dry ice were set at 2 locations on the property, including the woodpile that the patient had begun to move 6 days before becoming ill, to attract ticks. We also collected debris from the woodpile and processed the material by using Berlese funnels in an attempt to extract live ticks.

Results

Spirochetes in thin blood smears for the patient were visualized by staining with Giemsa and were positive by indirect fluorescent antibody staining with monoclonal antibodies H9724 and H9826, which identified the bacterium as *B. hermsii* (Figure 1). The spirochetes grew through 2 passages in mice and were isolated in the second passage in vitro culture. Stocks of the spirochete culture were frozen at –80°C, and the isolate was designated *B. hermsii* COR. MLST identified the isolate as *B. hermsii* that belonged to genomic group I (GGI) (*13*,*21*).

The patient lived on a rural, 40,469 m^2 area located east of Corvallis in Ravalli County, Montana, ≈12 km northeast of RML on the eastern slope of the Bitterroot Valley at an elevation of ≈1,250 m. The site included his house and a nearly pure stand of secondary growth yellow pine (*Pinus ponderosa*) and several large rock outcrops. We examined the site, which included cut yellow pine cones, feces, and shredded bark in the woodpile, and found evidence of rodent activity.

A total of 150 trap-nights captured 2 deer mice (*Peromyscus maniculatus*) and 7 yellow-pine chipmunks (*Tamias amoenus*), 1 of which was spirochetemic at the time of capture. Spirochetes in fixed thin blood smears were reactive with monoclonal antibodies H9724 and H9826, which also identified these bacteria as *B. hermsii*. Spirochetes in the chipmunk's blood were passed through 4 laboratory mice and isolated in the second passage by using in vitro culture. Stocks of this culture were also frozen at –80°C and the isolate was designated *B. hermsii* COC-807. In addition, a serum sample from 1 of the 6 remaining chipmunks was seropositive by immunoblot analysis with antibodies binding to numerous spirochete proteins and purified rGlpQ (Figure 2). MLST sequences for the same 7 loci for the spirochete isolated from the chipmunk were identical to sequences determined for the spirochete isolated from the patient.

We collected debris from the woodpile for processing by using Berlese funnels, and on the evening of August 6, 2013, we placed a CO_2 trap baited with a block of dry ice among the logs. The following morning, we found 3 *O. hermsi* nymphs in the substrate under the trap (Figure 3), and during the following weeks we found 6 more live ticks (4 nymphs, 2 males) from the debris that we processed by using the Berlese funnels. We fed 6 ticks individually on mice and triturated the remaining 3 ticks. The material from these 3 ticks was suspended in phosphate-buffered saline and injected into mice.

From the blood of these experimentally exposed mice, we isolated spirochetes that originated from 4 (2 nymphs, 2 males) of the 9 ticks. These isolates were established in culture, preserved as frozen stocks, and designated COT-5, COT-6, COT-7, and COT-8. MLST of the 7 loci identified COT-5 and COT-8 as *B. hermsii* belonging to GGI and identical to isolates from the patient and chipmunk; COT-7 belonged to GGII (7,111-bp sequence determined for this isolate). Isolate COT-6 was a mixed infection that demonstrated that 1 tick was infected with 2 *B. hermsii* strains that represented both genomic groups.

Once we identified the specific tick vector, we covered the woodpile with plastic tarpaulins and fumigated it with

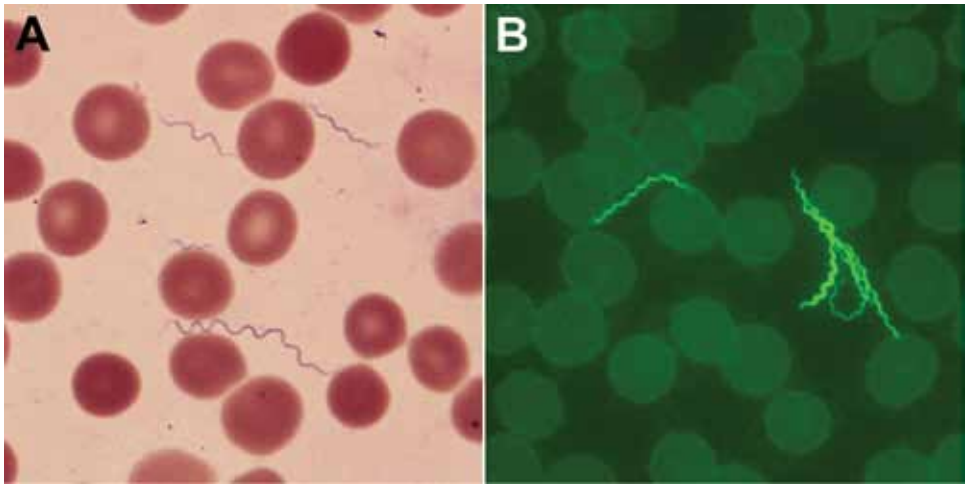

Figure 1. A) Spirochetes in blood smear of a 55-year-old man with tickborne relapsing fever, Bitterroot Valley, Montana, USA (Giemsa stain). Erythrocyte diameters are ≈6–8 μm. B) Spirochetes in blood smear of the patient visualized by indirect immunofluorescent antibody staining with mouse monoclonal antibody H9724 and goat anti-mouse antibody conjugated with fluorescein isothiocyanate (original magnification ×1,000).

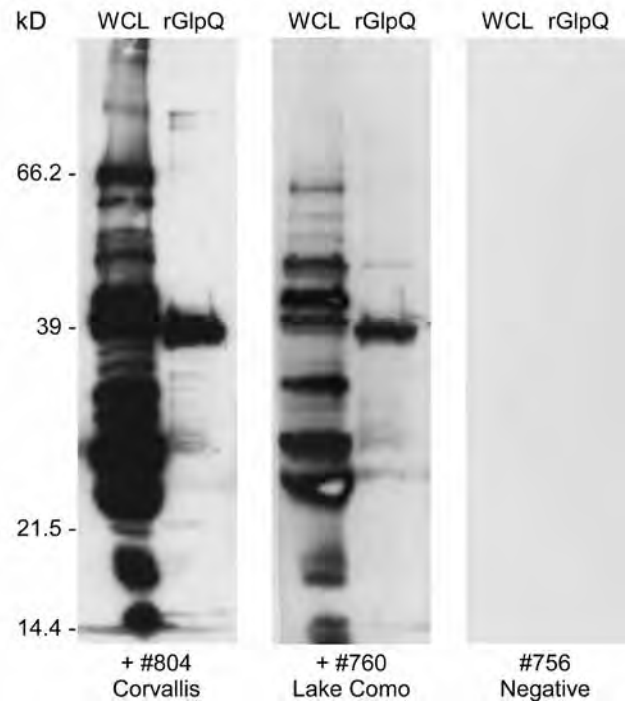

Figure 2. Immunoblot analysis of serum samples from 2 animals for *Borrelia hermsi*, Bitterroot Valley, Montana, USA. Samples were tested with *B. hermsii* whole cell lysates (WCL) (left lanes) and purified recombinant glycerophosphodiester phosphodiesterase (rGlpQ) (right lanes). +#804 Corvallis, seropositive sample from yellow-pine chipmunk (*Tamias amoenus*) trapped on patient's property; +#760 Lake Como, seropositive sample from red-tailed chipmunk (*T. ruficaudus*) trapped at Lake Como, Montana; #756 Negative, seronegative sample from chipmunk.

an aerosol from 2 cans of an over-the-counter insect fogger. This fogger contained 0.10% tetramethrin and 0.60% cypermethrin as the active ingredients.

At the time of the patient's illness, we had already begun a preliminary field study in the Bitterroot National Forest adjacent to the Bitterroot Valley to search for evidence that *B. hermsii* might be present. We investigated 2 sites, Lake Como (elevation 1,450 m) and Hughes Creek (elevation 1,575 m), which are ≈24 km and 72 km south-southwest of RML, respectively. At these 2 locations, we captured 8 species of rodents that included 178 animals (Table 2). None of the animals exhibited microscopically detectable spirochetes in their blood when captured. However, immunoblot analysis of serum samples demonstrated that 9 animals representing 4 species were seropositive, which indicated they had been previously infected with spirochetes. These animals included 1 red-tailed chipmunk (*Tamias ruficaudus*) at Lake Como (Figure 2) and 5 chipmunks of the same species at Hughes Creek. Additional seropositive animals at Hughes Creek included 1 northern flying squirrel (*Glaucomys sabrinus*), 1 golden-mantled ground squirrel (*Callospermophilus lateralis*), and 1 western jumping mouse (*Zapus princeps*).

On September 12, 2013, we collected soil litter from under a tick trap baited with dry ice at Hughes Creek, from which we extracted 1 *O. hermsi* nymph. This tick transmitted spirochetes when it fed on a laboratory mouse. We isolated spirochetes from the infected blood and designated this isolate HCT-4. MLST identified the spirochete as *B. hermsii* that belonged to GGI, and the spirochete was identical to the other GGI isolates described above. Sequences for the 16S rRNA, *flaB*, *gyrB*, *glpQ*, *vtp*, *fhbA*, and intergenic spacer loci for *B. hermsii* isolates COR, COC-807, COT-7, and HCT-4 have been deposited in GenBank under accession nos. KJ995774–KJ995801.

Discussion

This study reports an autochthonous human case of tick-borne relapsing fever caused by *B. hermsii* in the Bitterroot Valley of western Montana. The patient had many of the typical clinical signs and symptoms of this infection (*22*), with the notable exception of a unilateral uveitis that resulted in permanent damage to the right eye for which a corrective lens was required. Various forms of uveitis have been described with other spirochetal diseases, including Lyme disease (*23*), syphilis (*24*), and leptospirosis (*25*). Ocular complications, including uveitis, have been reported for cases of relapsing fever caused by other species of spirochetes (*16*), such as in troops in Libya during World War II (*26*). Iritis (anterior uveitis) has been associated with a few cases of relapsing fever in the southwestern United States (*27*), which were probably caused by *B. turicatae*. However, specific ocular involvement resulting from an infection with *B. hermsii* is rare.

Horton and Blaser (*28*) described endophthalmitis in a 3-month-old infant who contracted relapsing fever in a mountain cabin in Colorado. Although no spirochetes were isolated or identified, the ecologic and geographic setting suggests that the patient was infected with *B. hermsii*. The only case of uveitis purported to be caused by *B. hermsii* was in a 12-year-old boy exposed in eastern Oregon (*29*). Although no spirochetes were observed or identified in this patient, the clinical course and cross-reactive serologic test result for *B. burgdorferi*, a cause of Lyme disease, led the authors to conclude that the patient had been infected with *B. hermsii*. From the patient with uveitis in our study, we isolated the spirochete and confirmed its identity as *B. hermsii* by performing extensive molecular characterization. We are unaware that this etiologic confirmation was made for any case of relapsing fever with uveitis, regardless of the species of spirochete involved.

Our conclusion that the patient was infected locally is supported by his restricted travel just before onset of illness, our findings of infected ticks and an infected chipmunk

Figure 3. *Ornithodoros hermsi* nymph collected from the property of a 55-year-old man with tickborne relapsing fever, Bitterroot Valley, Montana, USA. Scale bar = 0.5 mm.

on his property, and extensive DNA sequence analysis that demonstrated that the isolates of *B. hermsii* from patient, chipmunk, and 2 of the ticks were identical. These results and our findings of seropositive animals and an infected *O. hermsi* tick south of the study site showed that the slopes of the Bitterroot Valley and surrounding areas represent a newly identified area to which *B. hermsii* spirochetes are endemic, which has the potential for being a source of human infections in this region of Montana.

Before our investigation, all known human cases of tick-borne relapsing fever caused by *B. hermsii* in Montana had originated on Wild Horse Island in Flathead Lake, in the northwestern part of the state. The first documented outbreak occurred there during the summer of 2002 (*10*,*30*). Five persons, all of whom resided elsewhere, became infected while sleeping in a tick-infested cabin during a family reunion. In 2004, three more persons became infected with *B. hermsii* while sleeping in another recreational cabin on the island, a short distance east of where the first outbreak occurred (*21*). Isolates of *B. hermsii* were obtained from the 2 patients infected in 2002 (*10*) and the 3 patients infected in 2004 (*21*). MLST of the isolates from these 5 patients showed that both genomic groups of *B. hermsii* were present on the island and in the same cabin (*21*).

We recently showed experimentally that 1 *O. hermsi* tick can become superinfected with spirochetes that belonged to both genomic groups and later transmit both types of bacteria simultaneously during a subsequent feeding (*31*). During our onsite investigation of the patient's property, we found 1 *O. hermsi* tick that was naturally infected with both genomic groups of spirochetes, which were co-transmitted during a single feeding on a mouse.

The patient with relapsing fever described in this report represents another example of an atypical exposure by becoming infected during a daytime activity. Although *O. hermsi* ticks are nocturnal and typically feed at night, persons who disturb materials infested with these ticks during the day might be bitten and become infected. We investigated a similar daytime exposure for a relapsing fever patient who was bitten by ticks while moving rodent-contaminated debris at Mount Wilson Observatory in Los Angeles County, California, USA (*20*). However, most persons in whom relapsing fever caused by *B. hermsii* develops are exposed at night in recreational cabins that are not the patient's primary residence.

Among wild rodents we sampled, 7 (70%) of 10 animals that were seropositive were chipmunks, and 1 yellow-pine chipmunk was infected with *B. hermsii* when captured. In other areas of the western United States, these animals play a major role as hosts for *O. hermsi* ticks and *B. hermsii* (*32*–*35*). Therefore, our observations extend considerably the geographic range for chipmunks involved in a natural enzootic focus of relapsing fever.

Residents and visitors to the Bitterroot Valley need to be alerted that there is the potential for becoming infected locally with the relapsing fever spirochete *B. hermsii*. Tickborne relapsing fever should be considered when patients seek treatment for a history of recurrent, acute febrile episodes. Confirmation of the infection is made most often by visualizing spirochetes in a stained, thin blood smear (Figure 1) (*36*) made during a febrile episode and examined by a trained medical technologist, as was performed for the patient in our study. In addition, this area of Montana has

Table 2. Animals serologically analyzed for infection with *Borrelia hermsi* in 3 locations, Bitterroot Valley, Montana, USA, July 8–September 13, 2013*

Species captured	Common name	No. captured by region			
		Corvallis	Hughes Creek	Lake Como	Total
Urocitellus columbianis	Columbian ground squirrel	0	53	0	53
Callospermophilus lateralis	Golden-mantled ground squirrel	0	2†	35	37
Tamias ruficaudus	Red-tailed chipmunk	0	28‡	9†	37
Tamias amoenus	Yellow-pine chipmunk	7†	1	0	8
Tamiasciurus hudsonicus	American red squirrel	0	5	0	5
Glaucomys sabrinus	Northern flying squirrel	0	1†	0	1
Peromyscus maniculatus	Deer mouse	2	37	2	41
Zapus princeps	Western jumping mouse	0	5†	0	5
Total	NA	9	132	46	187

*NA, not applicable.
†One animal was seropositive.
‡Five animals were seropositive.

long been a popular tourist destination for visitors from other regions of the United States, where the opportunity exists to enjoy many outdoor recreational activities. Health care providers in other parts of the country need to be aware that persons spending time outdoors in and around the Bitterroot Valley of Montana may be exposed to spirochetes causing relapsing fever in this newly identified disease-endemic area far from their place of residence.

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Simulation Study of the Effect of Influenza and Influenza Vaccination on Risk of Acquiring Guillain-Barré Syndrome

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It is unclear whether seasonal influenza vaccination results in a net increase or decrease in the risk for Guillain-Barré syndrome (GBS). To assess the effect of seasonal influenza vaccination on the absolute risk of acquiring GBS, we used simulation models and published estimates of age- and sex-specific risks for GBS, influenza incidence, and vaccine effectiveness. For a hypothetical 45-year-old woman and 75-year-old man, excess GBS risk for influenza vaccination versus no vaccination was –0.36/1 million vaccinations (95% credible interval –1.22% to 0.28) and –0.42/1 million vaccinations (95% credible interval, –3.68 to 2.44), respectively. These numbers represent a small absolute reduction in GBS risk with vaccination. Under typical conditions (e.g. influenza incidence rates >5% and vaccine effectiveness >60%), vaccination reduced GBS risk. These findings should strengthen confidence in the safety of influenza vaccine and allow health professionals to better put GBS risk in context when discussing influenza vaccination with patients.

Seasonal influenza vaccination programs have been implemented in many jurisdictions over the past 40 years. Although influenza vaccination has been shown to reduce influenza-associated illness and death (*1–3*), there is conflicting evidence about whether influenza vaccine may increase the risk of acquiring Guillain-Barré syndrome (GBS) (*4–14*).

GBS is a rare but serious autoimmune condition. Most cases in European and North American populations involve acute, inflammatory, demyelinating polyneuropathy. There are several less common forms of GBS, the most frequent of which are Miller Fisher syndrome and acute motor axonal neuropathy, which are more common in Asian and Latino populations (*15–17*). Most GBS patients require hospitalization, ≈25% experience acute respiratory failure requiring intensive care, 10% 20% are permanently disabled, and ≈4% die within 1 year of acquiring the condition (*15–17*). The risk for GBS is higher in males and with increasing age: the incidence in the general population ranges from a low of 0.45/100,000 person-years in girls <10 years of age to a high of 3.7/100,000 person-years in men >80 years of age (*18*).

Many GBS cases are preceded by a respiratory or gastrointestinal infection, most commonly caused by *Campylobacter jejuni* (*16*). Recent studies have provided evidence that influenza illness is associated with the development of GBS and that influenza vaccination may confer a much more modestly increased risk of GBS than that from influenza virus infection, but these findings are less consistent (*4–14*). The possible association between GBS and influenza vaccination is frequently cited as a reason for vaccine refusal by health care workers (*19–21*). This refusal occurs despite evidence that the risk of acquiring GBS is markedly higher from influenza illness than from influenza vaccination. For example, previous studies estimate that influenza illness may increase the risk for GBS by up to 16- to 18-fold, whereas influenza vaccination may only increase the risk by up to 2-fold (*4–14*) (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/21/2/13-1879-Techapp1.pdf).

On balance, it is unclear whether vaccination against seasonal influenza results in a net increase or decrease in the absolute risk of a person acquiring GBS. If influenza vaccination results in a small increased risk while reducing the incidence of influenza illness (which confers a much larger increase in GBS risk), then the net effect of vaccination could be a reduction in the absolute risk of GBS. The objective of this study was to assess, by using a simulation modeling approach, the effect of receipt of a seasonal influenza vaccine on a person's age- and sex-specific absolute risk of acquiring GBS.

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Table 1. Decision tree model inputs in a simulated study of the effect of influenza and influenza vaccination on the risk of acquiring GBS*

Parameter	Expected value (95% CI)	Range of values modeled in sensitivity analyses	References†
Relative risk for GBS from influenza vaccination	1.52 (1.17–1.99)	Fixed	(*11*), online Technical Appendix Table 1
Relative risk for GBS from influenza illness	15.81 (10.28–24.32)	Fixed	(*11*), online Technical Appendix Table 1
Joint risk of influenza vaccination and influenza illness	17.33 (additive)	15.81 (subadditive) to 24.03 (multiplicative)	No available data
GBS incidence rate	0.45–3.72 cases/100,000 person years‡	0.45 in youngest girls to 3.72 in oldest men	(*23*)
Influenza illness incidence rate	10% (base case)	2%–20%	See online Technical Appendix Table 2
Vaccine effectiveness		20%–80%	(*3, 25–27*)
<65 y of age	0.61 (0.30–0.52)		
≥65 y of age	0.50 (0.27–0.91)		

*GBS, Guillain-Barré syndrome.
†The online Technical Appendix is available at http://wwwnc.cdc.gov/EID/article/21/2/13-1879-Techapp1.pdf.
‡Depending on age and sex.

Methods

Intervention and Study Design

We compared the net impact of receiving a seasonal influenza vaccine versus not receiving the vaccine on a person's risk of acquiring GBS. We used a probabilistic decision tree modeling approach, in which each person was faced with the choice of receiving a vaccination against influenza (Figure 1). Using effect estimates and associated standard errors and/or confidence intervals from recent peer-reviewed literature, we simulated observations and modeled uncertainty by using appropriate distributional assumptions based on the type of effect estimate (e.g., relative risk [RR], incidence rate). We defined 2 base-case examples (a 45-year-old woman and a 75-year-old man) and then performed a series of sensitivity analyses to demonstrate the effect of important covariates on the risk for acquiring GBS.

Model Inputs

Age- and sex-specific baseline risks for GBS were based on a published regression model derived from a meta-analysis of studies reporting GBS incidence (*18*). We calculated age- and sex-specific individual GBS risk estimates for influenza incidence rates ranging from 2% to 20% and for estimates of vaccine effectiveness ranging from 20% to 80%. Published RR estimates for GBS with respect to influenza vaccination and influenza-like illness are listed in online Technical Appendix Table 1. We used the GBS risk estimates reported in Kwong et al. (*11*) in our simulation models; these estimates were consistent with findings of other studies (online Technical Appendix Table 1).

Given that influenza illness and vaccination are only 2 (relatively minor) transient contributors to the overall risk for GBS, we assumed that the risk increase for GBS persisted for only 6 weeks following exposure to both influenza illness and vaccination (*4*,*5*,*9*–*13*). A person's risk for seasonal influenza illness depends on age, vaccination status, geographic location, and sociodemographic factors. Estimates of influenza incidence also vary widely by year and case definition (i.e., confirmed by culture, PCR, or serologic testing). Published estimates of laboratory-confirmed influenza incidence in unvaccinated persons are listed in online Technical Appendix Table 2. Because of the observed annual variability in influenza incidence, we modeled a wide range (2%–20%) of rates in sensitivity analyses. Similarly, given that vaccine effectiveness varies by recipient age, type of vaccine (e.g., trivalent inactivated vaccine vs. live attenuated influenza vaccine), and success of matching the vaccine strains to circulating strains, we considered a range (20%–80%) of effectiveness estimates.

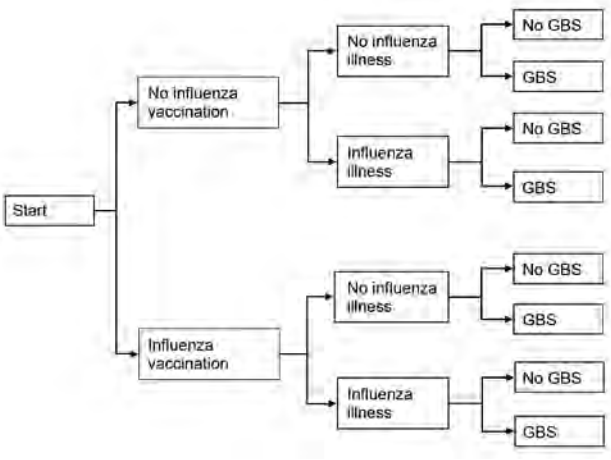

Figure 1. Probabilistic decision tree modeling approach used in a study simulating the effect of influenza and influenza vaccination on the risk of acquiring Guillain-Barré syndrome (GBS). It is assumed that each person has the choice of being vaccinated against influenza.

Table 2. Excess risk for GBS per million influenza vaccinations overall and for males and females separately by various influenza incidence rates in a simulated study*

Age, y, sex	ΔGBS risk (95% CrI), % ΔGBS risk ≤0, by influenza incidence rate†				
	2%	5%	10%	15%	20%
45					
Both	0.49 (−0.03 to 1.35), 3.5(+)	0.12 (−0.55 to 0.93), 35.7(±)	−0.48 (−1.63 to 0.37), 87.1(−)	−1.08 (−2.79 to −0.07), 98.2(−)	−1.69 (−3.99 to −0.43), 99.7(−)
F	0.37 (−0.02 to 1.01), 3.5(+)	0.09 (−0.41 to 0.70), 35.6(±)	−0.36 (−1.22 to 0.28),‡ 87.0(−)	−0.82 (−2.09 to −0.05), 98.2(−)	−1.28 (−2.98 to −0.33), 99.7(−)
M	0.66 (−0.05 to 1.79), 3.5(+)	0.16 (−0.74 to 1.24), 35.6(±)	−0.65 (−2.16 to 0.50), 87.1(−)	−1.46 (−3.69 to −0.09), 98.1(−)	−2.28 (−5.27 to −0.59), 99.7(−)
75					
Both	0.90 (0.19 to 2.71), 2.2(+)	0.43 (−0.79 to 2.20), 22.6(+)	−0.31 (−2.58 to 1.74), 64.7(±)	−1.07 (−4.55 to 1.51), 82.6(−)	−1.84 (−6.60 to 1.38), 89.3(−)
F	0.69 (0.02 to 2.18), 2.3(+)	0.32 (−0.61 to 1.77), 22.6(+)	−0.23 (−2.06 to 1.37), 64.7(±)	−0.80 (−3.67 to 1.16), 82.5(−)	−1.39 (−5.33 to 1.05), 89.3(−)
M	1.23 (0.02 to 3.90), 2.3(+)	0.58 (−1.09 to 3.15), 22.6(+)	−0.42 (−3.68 to 2.44),‡ 64.8(±)	−1.44 (−6.54 to 2.09), 82.6(−)	−2.48 (−9.47 to 1.89), 89.2(−)

*Assuming vaccine effectiveness of 61% for 45-year-old persons and 50% for 75-year-old persons. Assuming semi-additive effect whereby those vaccinated and who experience influenza illness experience the sum of the 2 relative risks (RR) (i.e., influenza illness RR = 15.81 + 1.52 = 17.33). A total of 1,000,000 simulations were conducted for each scenario. GBS, Guillain-Barré syndrome. Explanations for superscript symbols: (+) <25% of estimates have ΔGBS ≤0 (favors vaccination increasing GBS risk); (±) 25%–75% of estimates have ΔGBS ≤0 (neutral); (−) >75% of estimates have ΔGBS ≤0 (favors vaccination decreasing GBS risk).
†Absolute risk difference between vaccinated and unvaccinated persons. Negative values for Δ GBS indicate net reduction in no. of GBS cases in vaccinated vs. unvaccinated persons. The % of ΔGBS ≤0 is the percentage of simulation results where the absolute risk difference for vaccinated vs. unvaccinated was ≤0 (i.e., protective).
‡Base-case analyses.

Joint Effects of Influenza Vaccination and Illness

Because influenza vaccination may fail to prevent influenza illness, we considered 3 possible scenarios to model the joint effects of vaccination and influenza illness. If exposure to both influenza vaccination and illness occurred, it would be possible for the two 6-week risk periods to overlap. To simplify our simulation, we assumed a single 6-week exposure period for the 2 exposures combined. We varied the joint RR of influenza illness and vaccination to assess conditions of overlapping risk periods and interaction between exposures.

In the first scenario considered, we modeled exposures as independent and non-overlapping. Thus, the absolute risk of GBS would be equivalent to an additive joint effect on the RR scale: joint GBS risk = $RR_{(\text{influenza vaccination})}$ × (6-wk baseline GBS incidence rate) + $RR_{(\text{influenza illness})}$ × (6-wk baseline GBS incidence rate) = ($RR_{(\text{influenza vaccination})}$ + $RR_{(\text{influenza illness})}$) × (6 week baseline GBS incidence rate). We chose this additive joint risk model for our base-case simulations and then used different joint risk models in sensitivity analyses.

In the second scenario, we assumed the joint risk to be no higher than the risk of influenza illness alone: joint GBS risk = $RR_{(\text{influenza illness})}$ × (6-wk baseline GBS incidence rate). In the third scenario, we assumed that the joint risk was multiplicative on the RR scale: joint GBS risk = ($RR_{(\text{influenza vaccination})}$) × ($RR_{(\text{influenza illness})}$) × (6-wk baseline GBS incidence rate).

Modeling Approach

Technical details of our methodologic approach for generating representative simulated observations are provided in the online Technical Appendix. All simulation parameters and associated statistical uncertainty (i.e., standard errors) were chosen to reflect current peer-reviewed evidence. We simulated 1,000,000 observations each for vaccinated and unvaccinated persons and calculated the absolute risk difference with respect to GBS for the 2 scenarios. We then calculated the median and a 95% credible interval (CrI), defined as the region between percentiles 2.5 and 97.5 of the 1,000,000 simulated risk differences for each scenario. Point estimates for each of the model inputs were used to calculate absolute risk differences for deterministic sensitivity analyses.

Base-Case Analyses

We conducted 2 base-case analyses to represent the situation of typical persons faced with the decision of whether to receive the influenza vaccine. We first modeled the risk of GBS for a 45-year-old woman with a baseline risk for GBS of 0.97/100,000 person-years (95% CI 0.62–1.53) (*18*), a 10% chance of influenza illness (if unvaccinated), and vaccine effectiveness (if vaccinated) of 61% (95% CI 30%–52%) for a hypothetical trivalent inactivated vaccine (*3*,*22*). We then conducted a similar analysis for a 75-year-old man with a baseline risk for GBS of 3.07/100,000 person-years (95% CI 1.50–6.27) (*18*), a 10% chance of influenza illness (if unvaccinated), and a vaccine effectiveness (if vaccinated) of 50% (95% CI 27%–91%) for the same hypothetical vaccine (*23*).

Sensitivity Analyses

In sensitivity analyses, model inputs (influenza incidence rate, joint effect of vaccination and influenza illness on GBS risk, vaccine effectiveness, and sex) were changed

one at a time to determine the effect of higher and lower plausible values on excess risk for GBS with vaccination. We constructed tornado plots to display the effect of each factor we changed (*24*). The tornado plot displays the results of 1-way sensitivity analyses, which illustrate the effect of high and low values for each variable of interest while fixing all other variables at their respective base-case point estimates in the influenza GBS risk model. The tornado plots are displayed as stacked bar charts, with covariates ranked from most impactful at the top and the least impactful at the bottom, thus giving them the appearance of a tornado funnel. We then performed another series of sensitivity analyses involving 3-way sensitivity plots of excess risk for GBS by age (<18, 45, 60, and 75 years), vaccine effectiveness, and incidence of influenza illness, averaged over both sexes.

All expected values, measures of uncertainty for each, and ranges of inputs that we used in the base-case and sensitivity analyses are reported in Table 1. All simulations and statistical graphics were conducted in R version 3.0.1 (http://www.r-project.org/).

Results

Base-Case Analyses

When a 45-year-old woman was used as the base case, excess GBS risk for influenza vaccination versus no vaccination was calculated to be –0.36/1 million vaccinations (95% CrI –1.22 to 0.28), representing a small absolute reduction in GBS risk. Most (87%) of the simulated absolute risk differences were ≤0, indicating that vaccination is (slightly) protective against GBS. When a 75-year-old man was used as the base case, the excess GBS risk due to vaccination was estimated to be –0.42/1 million vaccinations (95% CrI –3.68 to 2.44), and 65% of simulated absolute risk differences were ≤0. Absolute risk differences are presented in Table 2 for influenza incidence rates ranging from 2% to 20%.

Sensitivity Analyses

In Figure 2, we present tornado plots that illustrate the relative influence of varying each model input (influenza incidence, vaccine effectiveness, sex, and joint RRs for vaccination and influenza illness) on the excess risk of GBS while holding all other factors fixed. For both base cases, influenza incidence and vaccine effectiveness were the most influential factors. Under most typical conditions, vaccination was protective against GBS; under conditions for which the model predicted an increased absolute risk, the excess GBS risk did not exceed approximately 1 in 1 million (Figure 2). The only exceptions noted were for the 75-year-old male base case when influenza incidence approached a low of 2% and when vaccine effectiveness was only 20%. For 45- and 75-year-old men and women (averaged over sex), when the influenza incidence rate was held constant at 10%, the threshold for protection from GBS with vaccination was crossed when the vaccine was at least 39% effective. Conversely, if vaccine effectiveness was held fixed at 61% in 45-year-old men and women and at 50% in 75-year-old men and women (*26*,*27*), the threshold for protection with vaccination was crossed when the influenza incidence rate was at least 6% and 7.5%, respectively. When the joint effect of influenza vaccination and illness were modeled as multiplicative on the RR scale, the benefit of vaccination on individual risk of GBS was muted or

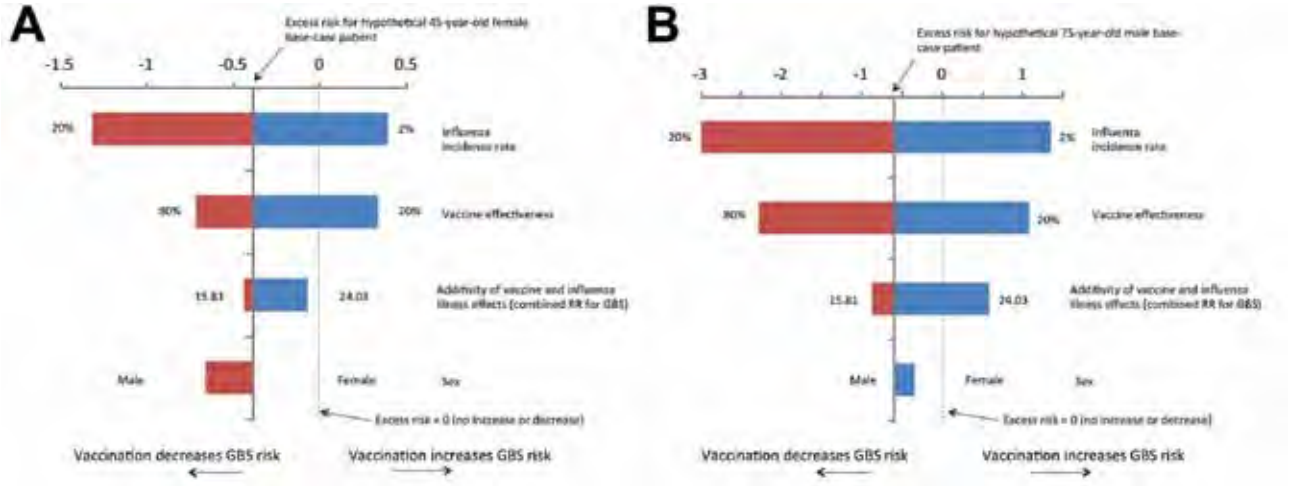

Figure 2. Sensitivity analyses for the excess risk of Guillain-Barré syndrome (GBS) per 1,000,000 influenza vaccinations. A) 45-year-old woman, assuming a 10% influenza incidence rate, 61% vaccine effectiveness, and combined relative risk (RR) of GBS of 17.33. B) 75-year-old man, assuming a 10% influenza incidence rate, vaccine effectiveness of 50% and combined RR of GBS of 17.33. Depending on the joint distribution of the probabilistic inputs to the simulation, these deterministic sensitivity analyses will not necessarily yield identical mean/median estimates to those from the probabilistic simulation for the same age, sex, and influenza incidence rate.

absent, compared with the benefit in the more plausible scenarios, in which 1) exposures were either independent and non-overlapping (additive joint effect on relative risk scale) or 2) joint exposure conferred the same risk as exposure to influenza illness alone (Figure 2).

In Figure 3, we separately present 3-way sensitivity analyses of absolute GBS risk by influenza incidence rate and vaccine effectiveness for each age group. When vaccine effectiveness was 60%, vaccination was protective in all age groups for influenza incidence rates of ≈6% or higher. Overall, the observed net benefit of vaccination on the risk for GBS was strongest with high vaccine effectiveness in older persons (for modeled scenarios in which vaccine effectiveness remained similar to that for younger persons) and among males because of their higher baseline incidence of GBS (not shown). For the lower limit of vaccine effectiveness of 20% considered for elderly subjects (ages 60 and 75, Figure 3, panels C, D), the excess risk of GBS was positive (favoring no vaccination) even for influenza incidence values as high as 20% (the highest incidence considered).

An online tool has been developed to allow readers to calculate individual GBS risk for a range of model inputs. The tool implements the deterministic model used to calculate the results presented in Figures 2 and 3 (http://www.stevenhawken.ca/Software_and_Web_Tools.html).

Discussion

To better understand the complex relationship between influenza vaccination and influenza illness with respect to GBS risk, we constructed probabilistic decision tree simulation models to evaluate the risk of GBS for a person who either does or does not receive seasonal influenza vaccine. Our simulations provide evidence that, under many conditions, vaccination is more likely to reduce rather than increase a person's overall risk of acquiring GBS. The most important factors in determining the net benefit or harm were the influenza incidence rate and vaccine effectiveness. Hence, when low influenza incidence was coupled with poor vaccine effectiveness, our models predicted a net increased risk of GBS with vaccination. Low influenza incidence and low vaccine effectiveness are not necessarily uncommon. For example, vaccine effectiveness tends to be lower in elderly persons, and incidence rates fluctuate from year to year. Where vaccine coverage and, by extension, herd immunity is high, influenza incidence rates will be lower. Even when both vaccine effectiveness and influenza incidence rates were low, the absolute risk increases observed in our simulations under

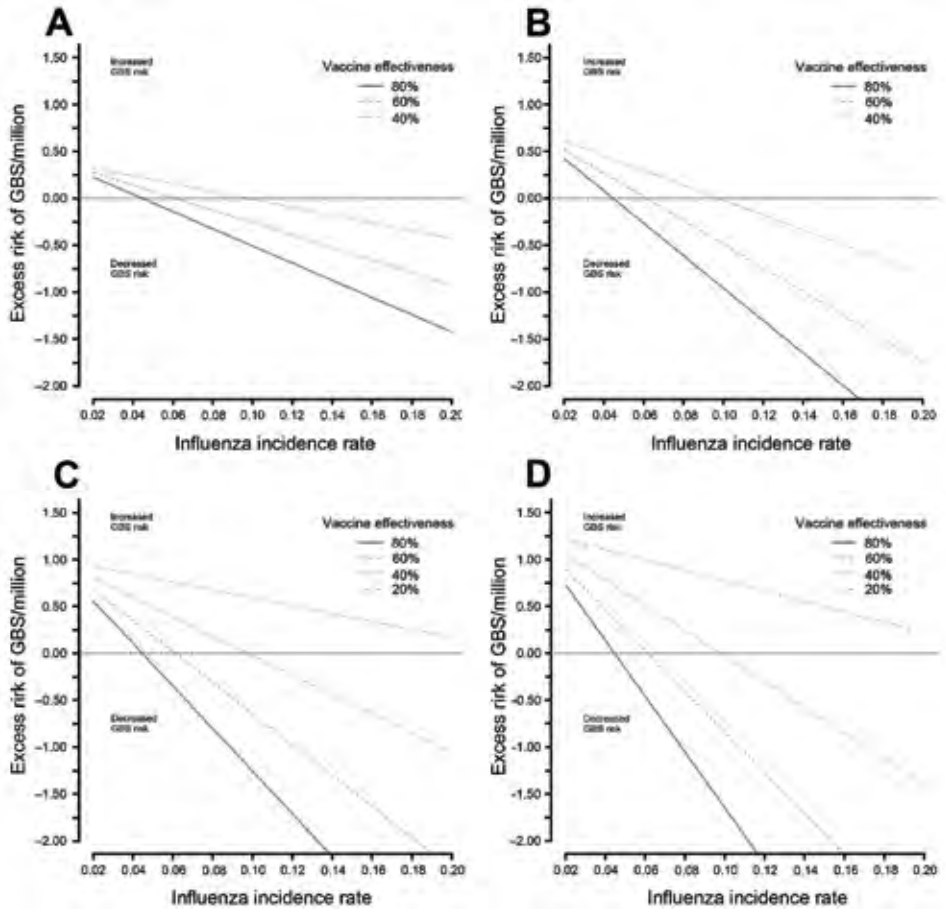

Figure 3. Excess risk of Guillain-Barré syndrome (GBS) per 1,000,000 influenza vaccinations by influenza incidence rate, age, and vaccine effectiveness for both sexes combined. A) Risk for persons <18 years of age; vaccine effectiveness of 40%–80%. B) Risk for persons 45 years of age; vaccine effectiveness of 40%–80%. C) Risk for persons 60 years of age; vaccine effectiveness of 20%–80%. D) Risk for persons 75 years of age; vaccine effectiveness of 20%–80%. Depending on the joint distribution of the probabilistic inputs to the simulation model, these deterministic sensitivity analyses will not necessarily yield identical mean/median estimates to those from the probabilistic simulation for the same age, sex, and influenza incidence rate.

these conditions were extremely small. In all but the most extreme cases, the excess risk with vaccination did not exceed the generally quoted figure of 1 in 1 million (*5*).

Although our investigation focused on individual risk, our results can potentially be interpreted from a population health perspective. For example, an excess risk of 1 in 1 million can be interpreted as the number of excess cases of GBS expected for every 1,000,000 people choosing to be vaccinated under the stated assumptions. The effect of incomplete vaccine uptake and herd immunity would serve to lower the effective number of persons vaccinated and lower the effective influenza incidence, which could be addressed in sensitivity analyses rather than by explicitly modeling them. These phenomena would have greatly complicated a simulation study focused at the population health level, but such complications were avoided in our study by focusing on individual persons.

Although local estimates of influenza incidence vary widely by year, age, geographic location, and sociodemographic factors, the World Health Organization estimates that overall, 20%–30% of children and 5%–10% of adults are affected by influenza illness annually (*28*). A recent meta-analysis reported seasonal influenza incidence rates of 5.4% (95% CI 3.0%–9.8%) in unvaccinated working adults, 24.2% (95% CI 15.1%–38.9%) in unvaccinated working adults living in households with children, and 18.7% (95% CI 15.8%–22.1%) in unvaccinated health care workers (*29*). This wide range of reported influenza incidence rates motivated our decision to use various influenza incidence rates to encompass the spectrum of plausible values.

Data from observational studies suggest that influenza vaccines may be less effective in elderly persons (*23*). This reduced effectiveness could result in a muted benefit of vaccination with respect to GBS risk, a fact that we confirmed in our simulations. However, higher baseline rates of GBS are observed in the elderly, so a vaccine with reduced efficacy could still be protective with respect to GBS risk. Furthermore, if vaccine effectiveness is reduced because of lowered immunogenicity (as distinct from poor antigen match), the risk for GBS from vaccination could be lowered if GBS risk is correlated with the immune response mounted by the vaccinee.

Vaccine effectiveness estimates are also heterogeneous by year and region and are dependent on antigen match between the vaccine and circulating virus strains. We found that when the annual influenza incidence was held fixed at 10%, vaccination was protective against GBS when vaccine effectiveness was at least 39%. Although Canadian annual adjusted vaccine effectiveness estimates from 2005–06 to 2010–11 have ranged from 37% (95% CI 17%–52%) to 61% (95% CI 26%–79%), estimates were ≥45% for every season except 2010–11 (*25*,*30*–*32*). A recent study from the United States reported a vaccine effectiveness of 60% (95% CI 53%–66%) for the 2010–11 season; the estimate for children 6 months to 8 years of age was 69%, and that for adults ≥65 years of age was 38% (*33*). For the 2011–12 season, a study from the United Kingdom reported a vaccine effectiveness of 23% (95% CI-10% to 47%) (*26*). For the same season, a European study reported estimates of 63% (95% CI 26%–82%) for persons 15–59 years of age and markedly lower estimates for younger (19%) and older (15%) persons (*34*). Despite their heterogeneity, these estimates of influenza incidence rates and vaccine effectiveness support our conclusion that under typical conditions, vaccination against seasonal influenza will result in a net decrease in absolute risk for GBS.

Previous studies of the risk for GBS from seasonal influenza vaccination and illness either looked solely at vaccination (*4*–*6*) or considered seasonal influenza illness and vaccination separately within the same study (*7*,*8*,*11*). Several studies looked at the risk for GBS from influenza A(H1N1) virus infection and vaccination (*27*,*35*,*36*). Our study considered the effect of seasonal influenza vaccination and influenza illness on GBS risk simultaneously, while taking into account the effect of vaccine effectiveness on reducing the incidence of influenza illness, as well as the important roles of age and sex on baseline risk for GBS, influenza incidence, and vaccine effectiveness.

This study has important strengths and limitations. A strength of our study is that we were able to model excess risk of GBS for a wide range of scenarios. We were also able to account for different assumptions about the combined effects of influenza illness and vaccination in modeling the joint risk of GBS if influenza illness were to occur in persons who had been vaccinated. We based all model inputs on recent peer-reviewed evidence.

One potential limitation of our study is that the principal studies quantifying the risk of GBS with influenza illness ascertained cases of influenza in different ways. Many of the studies reporting on vaccine safety and efficacy used medically attended, laboratory-confirmed influenza illness as the primary outcome (*3*,*22*,*23*,*37*). Estimates of GBS risk have been almost exclusively derived from studies of association with influenza-like illness; one exception is an ecologic study that did not provide an estimate of RR (*38*). It is unclear whether the risk for GBS (and influenza vaccine effectiveness) is similar for laboratory-confirmed influenza and influenza-like illness or influenza-coded health care encounters. Although the incidence of laboratory-confirmed influenza underestimates the true burden of influenza illness, influenza-coded outpatient visits are vulnerable to misclassification.

The excess risk calculated in our model is sensitive to the estimated combined effect of influenza illness and vaccination on the RR for GBS. At one extreme, we assumed

that the effects were multiplicative on the RR scale, and at the other extreme, we assumed the combined risk was no higher than the RR for influenza illness alone. It is possible that GBS risk varies by severity of illness, such that asymptomatic or minimally symptomatic illness confers a lower risk of GBS than more severe influenza-like illness; if this is the case, we may have overestimated influenza-associated GBS risk. It is also plausible that vaccination could reduce the severity of subsequent influenza illnesses that are not altogether prevented, and in turn, could reduce the risk of GBS due to those illnesses. Similarly, when vaccine effectiveness is reduced because of immunogenicity and not poor antigen match, the risk of GBS from vaccination could actually decrease if the risk is linked to the immune response mounted by the vaccinee. In our search of the literature, we did not find data on the severity of illness from influenza or other pathogens in relation to risk for GBS or on the combined risk of influenza vaccination and influenza illness on risk for GBS. Future studies exploring these questions would be welcome, although achieving the necessary statistical power would be extremely challenging.

In conclusion, our findings provide reassurance that influenza vaccination reduces individual risk of GBS except under conditions of low influenza incidence and/or low vaccine effectiveness. Even under those circumstances, in which the absolute risk of GBS may be raised by vaccination, the excess risk is small (in most cases, less than the generally quoted estimate of 1 in 1 million). The protective benefits of influenza vaccination are most pronounced for populations in which influenza incidence rates are higher (i.e., young children and the elderly, although effectiveness may be muted) and in those with a higher background risk for GBS (males and older persons). Influenza vaccination is an important population health intervention that reduces morbidity and mortality. Beyond these benefits, the tendency of influenza vaccination to reduce a person's overall risk of acquiring GBS under many conditions (although the absolute risk differences are extremely small) should strengthen confidence in the safety of influenza vaccination and allow health professionals to better put the risk of GBS in context when communicating risks and benefits to potential vaccinees.

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Evidence for *Elizabethkingia anophelis* Transmission from Mother to Infant, Hong Kong

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Elizabethkingia anophelis, recently discovered from mosquito gut, is an emerging bacterium associated with neonatal meningitis and nosocomial outbreaks. However, its transmission route remains unknown. We use rapid genome sequencing to investigate 3 cases of *E. anophelis* sepsis involving 2 neonates who had meningitis and 1 neonate's mother who had chorioamnionitis. Comparative genomics revealed evidence for perinatal vertical transmission from a mother to her neonate; the 2 isolates from these patients, HKU37 and HKU38, shared essentially identical genome sequences. In contrast, the strain from another neonate (HKU36) was genetically divergent, showing only 78.6% genome sequence identity to HKU37 and HKU38, thus excluding a clonal outbreak. Comparison to genomes from mosquito strains revealed potential metabolic adaptations in *E. anophelis* under different environments. Maternal infection, not mosquitoes, is most likely the source of neonatal *E. anophelis* infections. Our findings highlight the power of genome sequencing in gaining rapid insights on transmission and pathogenesis of emerging pathogens.

Microbial genome sequencing can enhance diagnosis and control of infectious diseases (*1*,*2*). Its ultimate molecular resolution is superior to other phenotypic and genotypic tests and enables not only rapid microbial identification but also characterization of transmission events. The technique has been applied in large-scale infectious disease outbreaks such as those caused by *Escherichia coli* O104:H4, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Enterococcus faecium*, *Pseudomonas aeruginosa*, *Vibrio cholerae*, and mycobacteria (*3*–*14*). However, the routine application of this method in diagnostic microbiology and infection control, especially for less well-defined, emerging pathogens, is yet to be explored.

Elizabethkingia anophelis is a recently discovered bacterium isolated from the midgut of the *Anopheles gambiae* mosquito in 2011 (*15*). The genus *Elizabethkingia* also includes *E. meningoseptica* (previously named *Chryseobacterium/Flavobacterium meningosepticum*) and *E. miricola* (*16*). *E. meningoseptica* causes neonatal sepsis and infections in immunocompromised persons. *E. anophelis* has also recently been reported to cause neonatal meningitis in the Central African Republic, and a nosocomial outbreak was reported in an intensive care unit in Singapore (*17*–*19*). However, the role of mosquitoes or other sources in the transmission of *E. anophelis* remains unclear.

In 2012, we encountered 3 cases of *Elizabethkingia* sepsis associated with meningitis in 2 neonates and chorioamnionitis in a neonate's mother in a hospital in Hong Kong. Three strains of *Elizabethkingia*-like, gram-negative bacilli sharing similar phenotypic characteristics were isolated from the 3 patients, but confident identification results were not obtained by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry and 16S rRNA gene sequencing. Moreover, clinical and microbiological data did not provide adequate clues about the possible transmission route. We therefore attempted to use draft genome sequencing to rapidly dissect transmission pathways and confirm the identity of the species.

Materials and Methods

Setting and Patients

The 3 patients were hospitalized in an acute regional hospital, Pamela Youde Nethersole Eastern Hospital, which is situated in the eastern area of Hong Kong Island. This study was approved by the Institute Review Board, Hospital Authority, Hong Kong (reference HKEC-2013-051).

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Microbiological Methods

Bacterial cultures and phenotypic identification were performed according to standard protocols by using the Vitek II system (bioMérieux, Marcy l'Etoile, France). Antimicrobial drug susceptibility testing was performed by E-test method for vancomycin and Kirby-Bauer disk diffusion for other drugs; because interpretative criteria for *Elizabethkingia* were lacking, results were interpreted according to Clinical and Laboratory Standards Institute for *Pseudomonas aeruginosa* (*20*). MALDI-TOF mass spectrometry was performed by the direct transfer method as described previously (*21*), with modifications by using the Bruker Daltonics microflex LT system with Reference Library Biotyper version 3.1 (Bruker Daltonik GmbH, Leipzig, Germany). Full 16S rRNA gene amplification and squencing were performed according to previously published protocols with modifications (*22*,*23*). Pulsed-field gel electrophoresis (PFGE) was performed by using the CHEF Mapper XA system (Bio-Rad, Hercules, CA, USA) and restriction endonuclease *Xba*I as described previously (*8*,*22*).

Draft Genome Sequencing and Analysis

The draft genome sequences of the 3 *E. anophelis* strains were determined by high-throughput sequencing with the Illumina HiSeq 2500 system (Illumina, San Diego, CA, USA). Samples of 50 ng of genomic DNA were extracted by using a genomic DNA purification kit (QIAGEN, Hilden, Germany) from cultures grown overnight on blood agar at 37°C, as described previously (*24*,*25*). Each sample was sequenced by 151-bp paired-end reads with mean library size of 350 bp. Sequencing errors were corrected by k-mer frequency spectrum analysis using SOAPec (http://soap.genomics.org.cn/about.html). De novo assembly was performed in SOAPdenovo2 (http://soap.genomics.org.cn/soapdenovo.html). Prediction of protein coding regions and automatic functional annotation was performed by using Glimmer3 (*26*) and the RAST (Rapid Annotations using Subsystem Technology) server (*27*). Antibiotic resistomes were identified by using the Antibiotic Resistance Genes Database (*28*). BLASTn comparisons were run in BLAST+ (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with an E-value cutoff of 10.0. In addition, manual annotation was performed on putative virulence and antibiotic resistance genes by protein domain predictions and multiple sequence alignments with orthologous genes. Intergenomic distance was calculated by using Genome-to-Genome Distance Calculator 2.0 (http://ggdc.dsmz.de/distcalc2.php) (*29*).

Results

Patients

In July 2012, a 21-day-old male neonate (patient 1) was admitted to Pamela Youde Nethersole Eastern Hospital for fever of 1 day's duration. He was born at the same hospital 21 days earlier at 41 weeks' gestation by vaginal delivery and was discharged on day 3. Physical examination did not show obvious infective focus. Serum C-reactive protein (CRP) was elevated to 109 mg/L. Lumbar puncture was performed; analysis of cerebrospinal fluid (CSF) showed polymorph pleocytosis, elevated protein levels, and low glucose levels (Table). Treatment was initiated for bacterial meningitis with empirical intravenous ampicillin and cefotaxime. Blood and CSF cultures recovered a gram-negative bacillus, designated HKU36. Antimicrobial drugs were changed to vancomycin, piperacillin, and rifampin on day 3. The patient was discharged after 3 weeks of intravenous drug treatment, without neurologic sequelae (Figure 1). The neonate's mother was admitted to the same hospital 1 day after the infant's admission for postpartum fever, chills, rigor, and abdominal pain. Transvaginal ultrasound showed no retained gestational products. Serum CRP level was elevated to 109 mg/L; however, blood cultures were negative. She was treated with intravenous cefuroxime and metronidazole and discharged on day 6 with oral cefuroxime and metronidazole.

In November 2012, a 33-year-old woman in week 30 of pregnancy (patient 2) was admitted to the same hospital because of prolonged premature rupture of membranes. She stayed at the same antenatal ward and in the same cubicle as the mother of patient 1 (Figure 1). Fever developed in the patient 3 days after admission, and clinical tests showed peripheral leukocytosis with neutrophilia (Table). Serum CRP was elevated to 108 mg/L. Treatment with intravenous penicillin G was commenced, and an emergency lower segment cesarean section was performed. Placental and uterine swab cultures recovered a gram-negative bacillus, designated HKU37. Blood cultures were negative. Antimicrobial drug treatment was changed to cefuroxime and metronidazole, followed by oral ciprofloxacin for 1 week. Her fever subsided, and she was discharged on day 8.

The baby girl (patient 3) of patient 2 was pale and flaccid at birth; apnea of prematurity developed, requiring cardiopulmonary resuscitation. Peripheral leukopenia and metabolic acidosis were also detected, and serum CRP level was elevated to 70.6 mg/L. Chest radiograph showed bilateral ground-glass appearance. Lumbar puncture was performed, and CSF showed lymphocytic pleocytosis with elevated protein levels and low glucose levels (Table). Ultrasound of the brain showed grade I to II intraventricular hemorrhages. Empirical intravenous ampicillin and cefotaxime at meningitic dose was started. Blood and CSF cultures recovered a gram-negative bacillus, designated HKU38. Antimicrobial drug therapy was changed to intravenous vancomycin, piperacillin/tazobactam, and rifampin on day 3, continuing for 3 weeks. Necrotizing enterocolitis and neonatal jaundice developed, but both resolved with

Table. Clinical characteristics and results of testing for 3 patients infected with *Elizabethkingia anophelis*, Hong Kong, 2012*

Characteristics	Patient 1	Patient 2†	Patient 3
Patient age/sex	21 d/M	33 y/F	0 d/F
Signs/symptoms	Fever	Fever, PPROM	Apnea at birth
Blood test results			
Total leukocytes, × 10^9 cells/L	16.0 (5.0–19.5)	15.2 (3.7–9.3)	5.1 (10.0–27.0)
Neutrophils, × 10^9 cells/L	6.8 (2.0–9.5)	12.5 (1.8–6.2)	1.2 (5.0–17.0)
Lymphocytes, × 10^9 cells/L	6.8 (2.5–11.0)	1.7 (1.0–3.2)	3.4 (3.0–10.0)
Monocytes, × 10^9 cells/L	2.3 (0.2–1.2)	0.8 (0.2–0.7)	0 (0.5–2.0)
Hemoglobin, g/dL	14.0 (11.0–19.0)	10.7 (11.5–15.4)	16.1 (13.5–19.5)
Platelets, × 10^9/L	180 (180–460)	241 (160–420)	186 (100–300)
C-reactive protein, mg/L	109 (<8.0)	108 (<5.0)	70.6 (<8.0)
CSF test results			
Total leukocytes, × 10^6 cells/L	1,445	NA	5,850
Polymorphs, %	67	NA	1
Lymphocytes, %	33	NA	99
Protein, g/L	1.33 (0.15–0.45)	NA	2.69 (0.15–0.45)
Glucose, mmol/L	2.2 (2.8–4.4)	NA	1.5 (2.8–4.4)
CSF/serum glucose, %	38	NA	24
Positive culture sites for *E. anophelis*	Blood, CSF	Placental swab, uterine swab	Blood, CSF
Phenotypic characteristics of isolates			
Colony pigment	Pale yellow	None	None
Citrate utilization	Negative	Delayed positive	Delayed positive
Antimicrobial drug susceptibilities of isolates			
Ampicillin	Resistant	Resistant	Resistant
Pipercillin	Susceptible	Susceptible	Susceptible
Cefoperazone/sulbactam	Susceptible	Susceptible	Susceptible
Cefotaxime	Intermediate	Resistant	Resistant
Ceftazidime	Resistant	Resistant	Resistant
Imipenem	Resistant	Resistant	Resistant
Amikacin	Resistant	Resistant	Resistant
Gentamicin	Resistant	Resistant	Resistant
Kanamycin	Resistant	Resistant	Resistant
Streptomycin	Resistant	Resistant	Resistant
Tobramycin	Resistant	Resistant	Resistant
Ciprofloxacin	Susceptible	Susceptible	Susceptible
Moxifloxacin	Susceptible	Susceptible	Susceptible
Tetracycline	Resistant	Resistant	Resistant
Trimethoprim/sulfamethoxazole	Susceptible	Susceptible	Susceptible
Rifampin	Susceptible	Susceptible	Susceptible
Chloramphenicol	Resistant	Resistant	Resistant
Vancomycin MIC, μg/mL	16	4	4
Antimicrobial drug regimen	Ampicillin + cefotaxime; vancomycin + piperacillin + rifampin	Penicillin G; cefuroxime + metronidazole; ciprofloxacin	Ampicillin + cefotaxime; vancomycin + pipercillin/tazobactam + rifampin
Complications	None	None	Respiratory distress, intraventricular hemorrhage

*Reference ranges are shown in parentheses. PPROM, prolonged premature rupture of membranes; CSF, cerebrospinal fluid.
†Mother of patient 3.

treatment (Figure 1). The infant was discharged on day 54 without neurologic sequelae.

Clinical and Microbiological Investigations

The 3 isolates from these patients, HKU36–38, were nonmotile, oxidase-positive, non–glucose-fermenting, gram-negative bacilli. Their phenotypic characteristics are summarized in the Table and online Technical Appendix Table 1 (http://wwwnc.cdc.gov/EID/article/21/2/14-0623-Techapp1.pdf). The isolates were identified as *E. meningoseptica* by using the Vitek II identification system (bioMérieux, Marcy L'Étoile, France). However, MALDI-TOF mass spectrometry identified strains HKU37 and HKU 38 as *E. meningoseptica* (best match to *E. meningoseptica* strain 002_NEB14 NFI, with scores of 2.106 and 2.007, respectively), whereas strain HKU36 was only identified to the genus level as *Elizabethkingia* species (best match to *E. meningoseptica* strain 002_NEB14 NFI, with score of 1.853) (online Technical Appendix Figure 1). The isolates' 16S rRNA gene sequences exhibited 99.1%–99.9% nucleotide identities to those of *E. anophelis* type strain R26^T (GenBank accession no. EF426425) and 97.4%–99.9% nucleotide identities to those of *E. meningoseptica* strains deposited in GenBank (GenBank accession nos. HM056770.1, GU180602.1, JQ673498.1, FJ816020, AVCQ01000012, FJ839441.1, JN201943.1, and AJ704540).

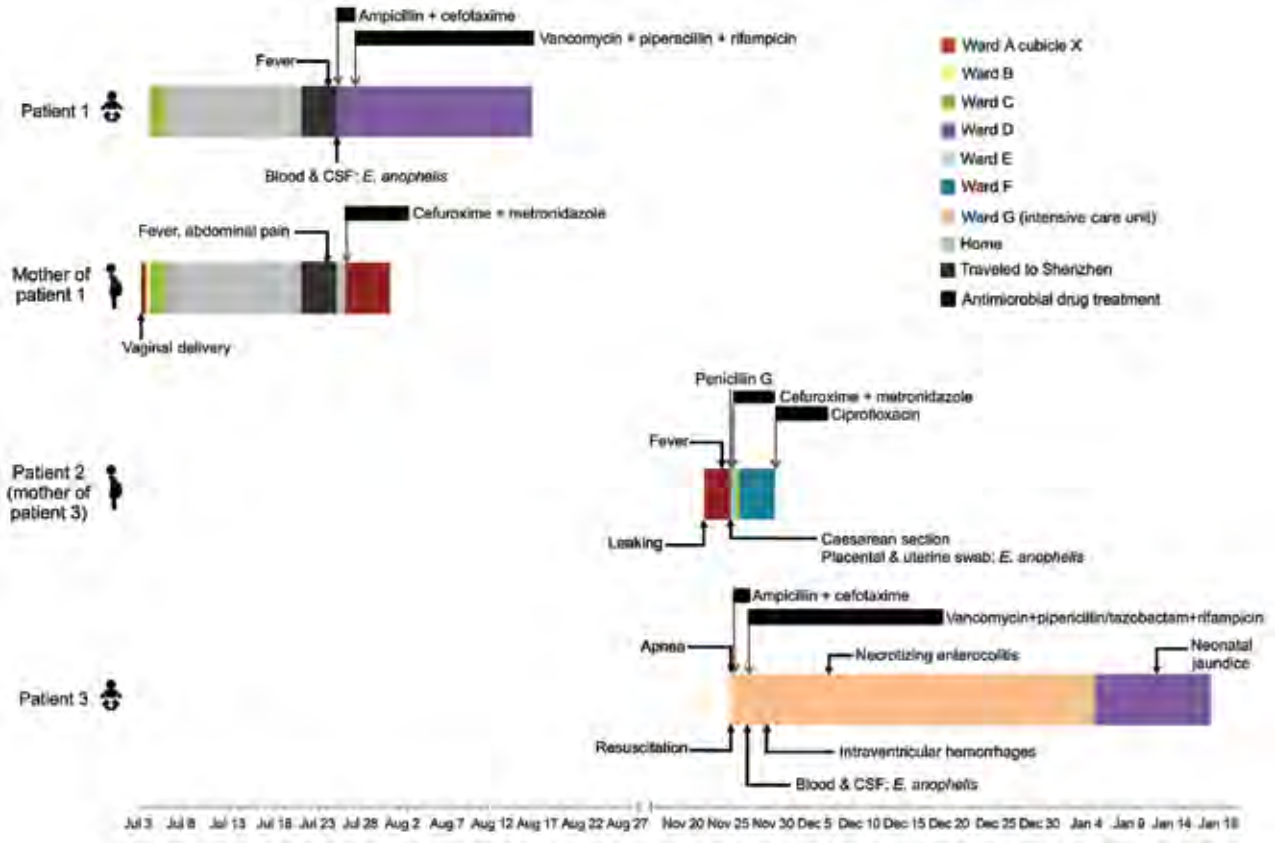

Figure 1. Clinical course of illness in 3 patients infected with *Elizabethkingia anophelis* in whom sepsis developed and the mother of patient 1, who had culture-negative postpartum fever, Hong Kong, 2012. Locations where patients were treated at the hospital and times when they were home are noted. CSF, cerebrospinal fluid; leaking, leaking of amniotic fluid (membrane rupture).

The high sequence identities to both *E. anophelis* and *E. meningoseptica* made the species identity of the 3 strains uncertain, despite 16S rRNA gene sequencing. Moreover, the strains exhibited minor differences in phenotypes and antibiogram (Table). Further, because the mothers stayed in the same ward before delivery (although 4 months apart), concerns of a possible nosocomial outbreak were raised. However, environmental and water samples from the hospital and patients' homes were culture-negative for *E. anophelis*. A program of enhanced infection control measures was enforced in the hospital, and no further cases were identified.

Genome Sequencing and Comparative Analysis of *E. anophelis* Genomes

We sequenced the draft genomes of strains HKU36–38 to investigate their genetic relatedness and confirm their species identity. Sequencing generated 11–15 million paired-end reads per strain (estimated 410–540-fold coverage). After de novo assembly, the 3 draft genomes ranged from 3.92–3.99 Mb in length (G + C content 35.4%–35.8%) and were distributed in 42–52 large (>500 bp) contigs (EMBLaccessionnos.CBYD010000001–CBYD010000042, CBYE010000001–CBYE010000032, CBYF010000001–CBYF010000038; online Technical Appendix Table 2). These contigs contained 3,654–3,667 predicted protein-coding genes (Figure 2, panel A). Using Genome-to-Genome Distance Calculator for intergenomic distance estimation, which enabled genome-based species delineation analogous to traditional DNA–DNA hybridization method, we found that these genomes shared 78.3%–85.4% nucleotide identities to the draft genome sequence of *E. anophelis* type strain R26^T, the initial isolate from an *Anopheles gambiae* mosquito (GenBank accession no. NZ_ANIW00000000.1). However, the genomes shared only 23.6%–23.7% nucleotide identities to the draft genome sequence of *E. meningoseptica* type strain ATCC 13253^T (GenBank accession no. BARD00000000.1) (Figure 2, panel B). Phylogenetic analysis using the draft genomes and concatenated sequences of 69 housekeeping genes also supported the identification of the 3 strains as *E. anophelis* (Figure 3; online Technical Appendix Figure 2).

The sequences from 52 contigs of strain HKU37 demonstrated 99.4% nucleotide identity to those from 46 contigs of strain HKU38, indicating that these draft genomes

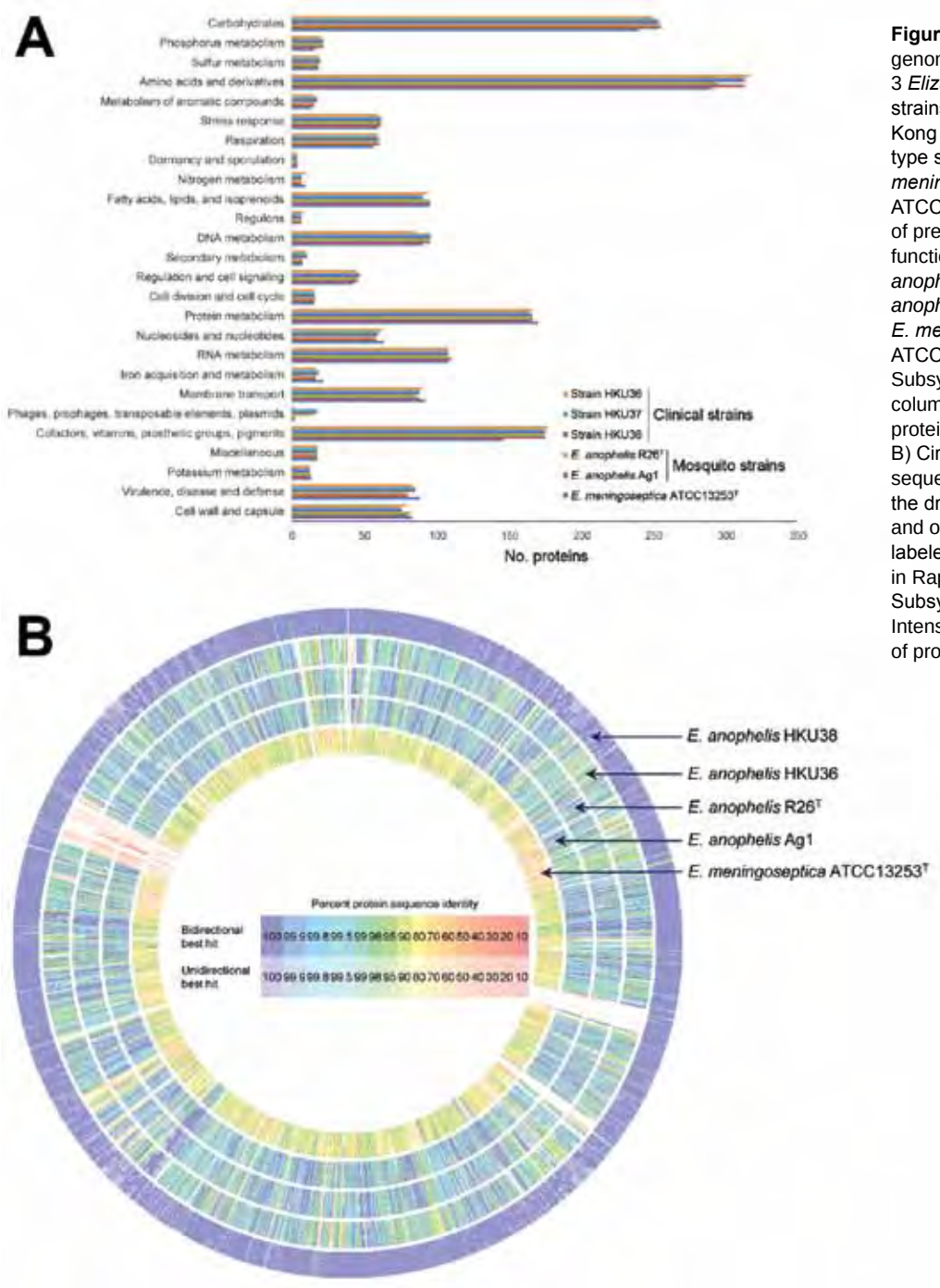

Figure 2. Comparison of draft genome sequence data of the 3 *Elizabethkingia anophelis* strains from patients in Hong Kong (HKU36–38), *E anophelis* type strain R26T, and *E. meningoseptica* type strain ATCC 13253T. A) Distributions of predicted coding sequence function in genomes of *E. anophelis* strains HKU36–38, *E. anophelis* type strain R26T, and *E. meningoseptica* type strain ATCC 13253T according to SEED Subsystems are shown. The columns indicate the number of proteins in different subsystems. B) Circular representation of sequence comparison between the draft genome of strain HKU37 and other draft genomes as labeled. Comparison generated in Rapid Annotations using Subsystem Technology (27). Intensity of color indicates degree of protein identity.

are essentially identical (Figure 2, panel B, and Figure 3). The small intergenomic distance can be explained by slight differences in coverage or contig assembly; sequences of 2,000 high-coverage protein-coding genes were identical between HKU37 and HKU38. In contrast, these sequences demonstrated only 78.6% nucleotide identity to those from the 42 contigs of strain HKU36, indicating that strain HKU36 is genetically divergent (Figure 2, panel B, and Figure 3), consistent with PFGE patterns (Figure 4). Moreover, a potential genetic island consisting of conjugative transposable elements was found in strains HKU37 and HKU38 but not in HKU36. Our results exclude a clonal outbreak, but the extremely close genetic relatedness between strains HKU37 and HKU38 provides evidence for vertical transmission from patient 2 to patient 3 (mother to infant).

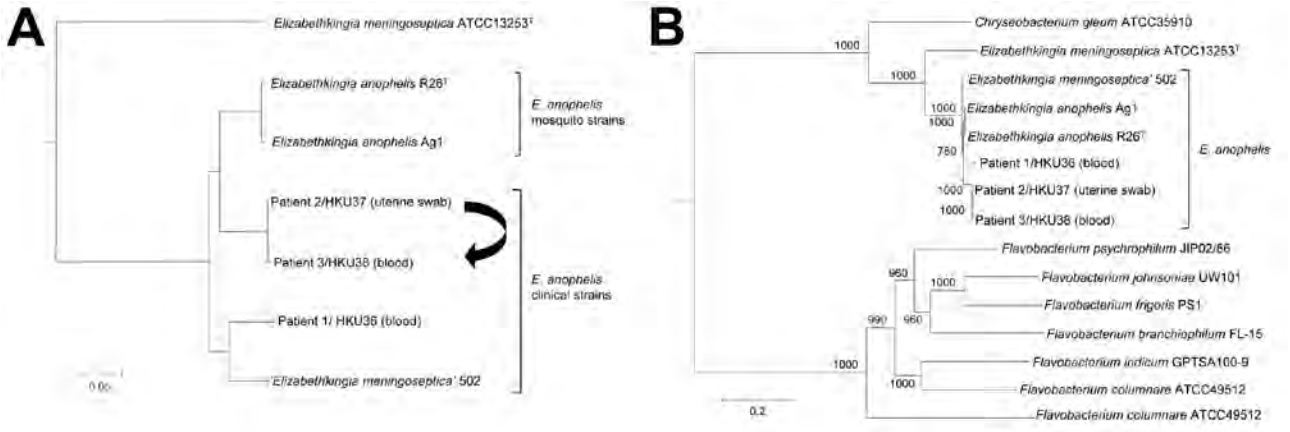

Figure 3. Phylogenetic trees constructed by using draft genome sequences and concatenated sequences of 69 housekeeping genes of 3 *Elizabethkingia anophelis* strains from patients in Hong Kong (HKU36–38). A) Neighbor-joining tree constructed on the basis of draft genome sequences using by using Genome-to-Genome Distance Calculator 2.0 (http://ggdc.dsmz.de/distcalc2.php; formula 1) and *Chryseobacterium gleum* ATCC 35910 as the root. Arrow indicates route of mother-to-neonate transmission. B) Maximum-likelihood tree constructed on the basis of 69 housekeeping genes, showing the relationship of *E. anophelis* strains HKU36–38 to related bacterial species, using RAxML version 7.2.8 (http://sco.h-its.org/exelixis/software.html) and *Weeksella virosa* DSM 16922 as the root. A total of 78,520 nt positions were included in the analysis. Bootstrap values were calculated from 1,000 replicates. Scale bars indicate mean number of nucleotide substitutions per site on the respective branches. Gene names and accession numbers are given as cited in GenBank (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/21/2/14-0623-Techapp1.pdf). '*E. meningoseptica*' strain 502 is a misidentified isolate that actually belongs to *E. anophelis* on the basis of draft genome sequencing.

Potential Virulence Factors and Resistance Genes in *E. anophelis*

The association of *E. anophelis* with neonatal meningitis in this and previous reports (*17*,*18*) suggests that the bacterium may possess virulence factors that enable it to invade the central nervous system. The 3 draft genomes we identified contain homologs of several virulence genes found in *Listeria monocytogenes*, which also causes neonatal meningitis. These genes include cell wall hydrolase A, which enables host cell invasion; phosphatidylinositol-specific phospholipase (PlcA) and listeriolysin O (LLO), which enable escape from the primary vacuole of macrophages, and genes that enable survival in the secondary vacuole of macrophages; and virulence cluster protein B (VclB). Phosphatidylinositol-specific phospholipase, listeriolysin O, and virulence cluster protein B are located in the *Listeria* pathogenicity island LIPI-1 (*30*,*31*). Moreover, the 3 genomes we identified contain homologs of arylsulfatase and genes that enable invasion of brain endothelial cells, which contribute to the ability of *Escherichia coli* to cross the blood–brain barrier in neonatal meningitis (*32*).

Vertical transmission of *E. anophelis* from mother to infant also suggests that the bacterium may be able to colonize the vagina before causing ascending chorioamnionitis in the mother and neonatal infection through transplacental spread. A homologof the gene encoding agmatine deiminase, AgDI, which mediates acid tolerance in *L. monocytogenes* (*33*), was found in the *E. anophelis* genomes. Further studies may investigate the possible role of AgDI and potential adherence factors for vaginal colonization in *E. anophelis*.

Similar to *E. meningoseptica*, the 3 *E. anophelis* isolates we identified are resistant to multiple antimicrobial drugs. We found various antimicrobial resistance genes consistent with their resistance phenotypes, including metallo-β-lactamase (bla_{GOB-1} and *blaB14* in strain HKU36 and a novel bla_{GOB} and *blaB1* in strains HKU37 and HKU38) and extended-spectrum β-lactamase ($blaA_{CME-1}$ in strains HKU37 and HKU38 and a potential novel $blaA_{CME-1}$ variant in strain HKU36). A comparison of these β-lactamases to their corresponding orthologs in *E. meningoseptica* genomes revealed only 74%–85% amino acid identities, indicating that *E. anophelis* and related bacteria are potential reservoirs of novel β-lactamase genes (*19*,*34*,*35*). Other antimicrobial resistance genes found included multidrug-resistance efflux pumps (ATP binding cassette superfamily, major facilitator superfamily, resistance-nodulation-division families, multidrug and toxic-compound extrusion family) that potentially carry resistance to a variety of compounds; chloramphenicol acetyltransferase; aminoglycoside 6-adenyltransferase; and tetracycline resistant gene. Moreover, a putative *tetX* gene was also identified; this gene encodes a predicted flavin-dependent monooxygenase with tetracycline/tigecycline-degrading activity, although the 3 strains we identified are only resistant to tetracycline but remained susceptible to other related drugs, including tigecycline.

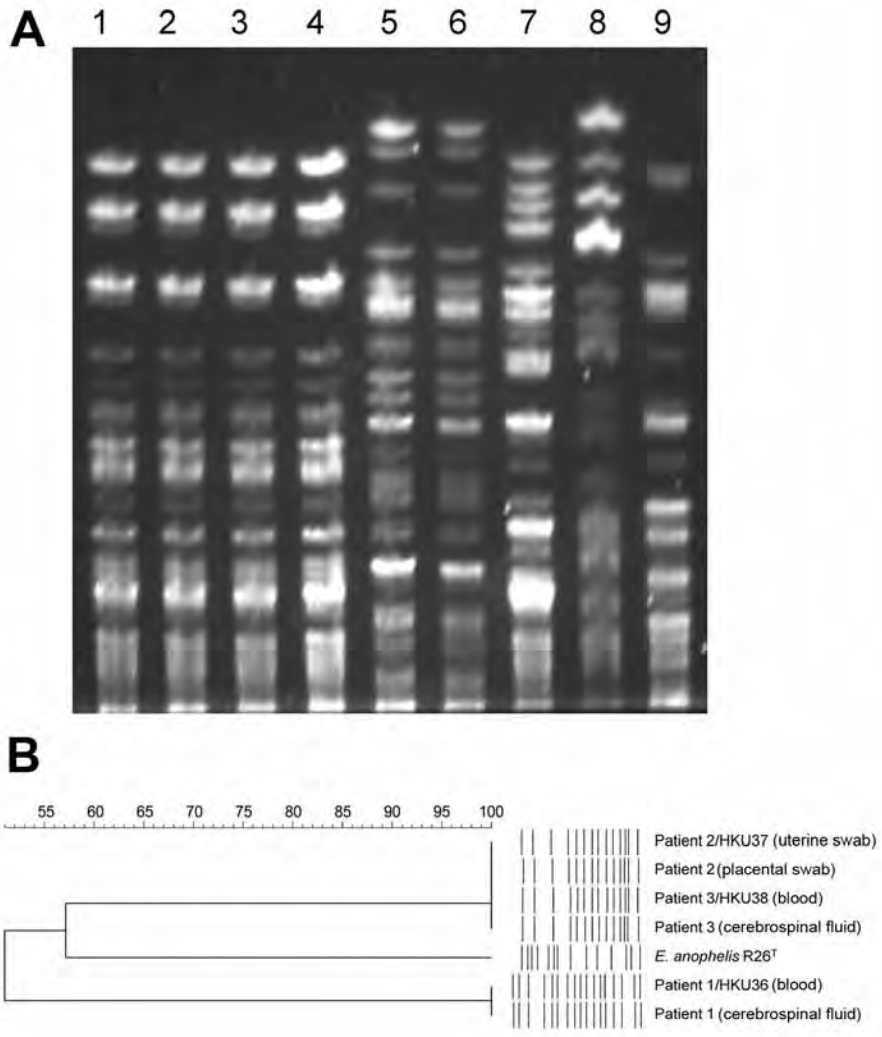

Figure 4. Pulsed-field gel electrophoresis (PFGE) analysis of samples from patients in Hong Kong showing 3 *Elizabethkingia anophelis* strains compared with reference *Elizabethkingia* isolates. A) PFGE performed by using CHEF Mapper XA system (Bio-Rad, Hercules, CA, USA) and restriction endonuclease XbaI shows that isolates from patient 2 and patient 3 are indistinguishable, wheras isolates from patient 1 possess distinct PFGE patterns. Lane 1, *E. anophelis* strain HKU37 from uterine swab specimen of patient 2; lane 2, placental swab specimen from patient 2; lane 3, *E. anophelis* strain HKU38 from blood of patient 3; lane 4, cerebrospinal fluid from patient 3; lane 5, *E. anophelis* strain HKU36 from blood of patient 1; lane 6, cerebrospinal fluid from patient 1; lane 7, *E. anophelis* type strain R26^T; lane 8, *E. meningoseptica* type strain ATCC 13253^T; lane 9, *E. miricola* type strain LMG22470T. B) Dendrogram constructed with PFGE data by similarity and clustering analysis using the Dice coefficient (1% tolerance and 0.5% optimization) and the unweighted pair-group method using average linkages with GelCompar II (Applied Maths, Sint-Martens-Latem, Belgium).

Comparison of Genomes from Human and Mosquito *E. anophelis* Strains

E. anophelis strains R26^T and Ag1 were isolated from mosquitoes (*35*). Compared with those strains, the genomes of the 3 strains we identified possessed 33 unique hypothetical proteins. Moreover, the genetic island consisting of conjugative transposable elements found in strains HKU37 and HKU38 was also absent in the mosquito strains. In contrast to the mosquito strains, which possessed genes encoding for xylose isomerase (XylA) and xylulose kinase (XylB), these 2 genes were absent in the 3 strains we identified. This finding may reflect different requirements for sugar metabolism in *E. anophelis* under different environments. Notably, despite the presence of XylA and XylB, *E. anophelis* mosquito strain R26^T did not produce acid from xylose (*15*). However, this finding does not exclude the strain's ability to metabolize xylose, as D-xylulose 5-phosphate, the product of XylA and XylB, can be used as a substrate for the pentose-phosphate pathway. XylA and XylB were also absent in the genome of *E. meningoseptica* type strain ATCC 13253^T, which suggests that mosquito strains of *E. anophelis* may be evolutionarily distinct from clinical strains of *E. anophelis* and *E. meningoseptica*. More genome sequence data from other clinical and environmental strains of *E. anophelis* may shed light on the ecology, biology, and pathogenesis of *E. anophelis*.

Discussion

This study demonstrates the power of draft genome sequencing to rapidly dissect transmission pathways for emerging bacterial infections. Our results showed that vertical perinatal transmission had occurred from patient 2, a pregnant woman who had chorioamnionitis, to patient 3, a neonate who had early onset neonatal meningitis. The infective source for patients 2 and 3 was unlikely to have been patient 1 or his mother. However, we speculate that the mother of patient 1 might also have had *E. anophelis* chorioamnionitis, as evidenced by postpartum fever and

abdominal pain, which resulted in late-onset meningitis in her son owing to fastidious bacterial growth. Although strain HKU36 did not belong to the same clone as strains HKU37/38, a polyclonal outbreak of *E. anophelis* sepsis in the labor ward, in which case an environmental source is likely, could not be excluded.

The discovery of *E. anophelis* in mosquito gut has raised suspicion that mosquitoes are the source of neonatal meningitis cases in Africa (*17*). Although *Anopheles* mosquitoes are not found in Hong Kong, the role of local mosquitoes as reservoirs for *E. anophelis* remains unknown. Nonetheless, the vertical transmission demonstrated in 1 neonate makes mosquitoes unlikely as vehicles of transmission in our cases.

Our report provides genomic evidence for vertical transmission in neonatal meningitis. Whereas we cannot ascertain how the mother(s) acquired the infection, our results prompt further work to assess the importance of maternal source in neonatal meningitis caused by *E. anophelis* and other bacterial agents. Maternal colonization with Lancefield group B streptococcus (GBS) during pregnancy is the primary risk factor for early onset neonatal disease. However, direct microbiological evidence for vertical transmission is seldom available, especially for bacterial agents other than GBS. Further genomic studies may help investigate the role of vertical transmission in neonatal meningitis caused by other bacteria. Current indications for intrapartum antimicrobial drugs prophylaxis have been determined on the basis of risk factors for early onset GBS disease; therefore, intravenous penicillin G or ampicillin is often the standard empirical regimen used. However, if further research determines that the mother may also be a source of transmission for other bacterial agents, broader-spectrum antimicrobial drugs may need to be considered as treatment for intrapartum fever or prolonged rupture of membranes.

E. anophelis is likely an underreported bacterium because it can be easily misidentified as *E. meningoseptica*, which shares a similar phenotypic profile (*17*,*19*). The *E. anophelis* isolates from the recent outbreak reported in Singapore were initially mistakenly identified as *E. meningoseptica* (*19*,*36*). Of the 3 strains we identified, 2 were misidentified as *E. meningoseptica* with MALDI-TOF mass spectrometry, the state-of-the-art technology, which is replacing conventional phenotypic identification in diagnostic laboratories. The reason for failure of MALDI-TOF mass spectrometry to identify these strains was that reference *E. anophelis* strains are lacking in existing diagnostic spectrum databases, as is the case with other less commonly encountered organisms (*21*).

Although 16S rRNA gene sequencing should provide sufficient resolution, some strains indexed as *E. meningoseptica*, such as strains G3-1-08 and 502, were actually more closely related to *E. anophelis* than to *E. meningoseptica* in their 16S rRNA sequences (Figure 3; online Technical Appendix Figure 2) (*37*). These ambiguous, potentially misidentified strains may cause incorrect interpretations in suspected *E. anophelis* infections. For example, the sequence of strain HKU36 possessed 99.8% nucleotide identity to that of *E. meningingoseptica* strain G3-1-08 but only 99.1% nucleotide identity to that of *E. anophelis* strain R26^T. Furthermore, phenotypic tests such as acid production from cellobiose and citrate utilization, previously proposed to be useful for identification of *E. anophelis* (*15*), are probably unreliable in differentiating among *Elizabethkingia* species. For example, *E. anophelis* strain R26^T produces acid from cellobiose, but the 3 strains we identified do not; in addition, *E. anophelis* strains R26^T, HKU37, and HKU38, but not strain HKU36, utilize citrate (online Technical Appendix Table 1). Strain HKU36 displayed higher MIC of vancomycin than did strains HKU37 and HKU38 and type strains of *E. anophelis*, *E. meningoseptica*, and *E. miricola*, which correlates with previous reports on variable vancomycin susceptibilities in *Elizabethkingia* (*38*,*39*). The species identity of the 3 strains we identified was only resolved by intergenomic comparison. Inclusion of *E. anophelis* in MALDI-TOF MS databases and rectification of 16S rRNA gene sequences of *Elizabethkingia* strains deposited in databases will enable accurate diagnosis of more *E. anophelis* infections.

The draft genome sequences we identified have enabled rapid exploration of novel β-lactamase and other antimicrobial drug resistance genes and possible virulence genes in *E. anophelis*, highlighting the potential of genome sequencing in identifying novel drug-resistance mechanisms and guiding treatment regimens for emerging, multidrug-resistant bacteria (*25*,*34*,*40*). Because previous cases of *E. anophelis* neonatal meningitis have been associated with poor outcomes (*17*,*18*), further work to elucidate the pathogenesis and antimicrobial drug resistance patterns of this emerging pathogen may help improve clinical management of illness. The findings of potential genes related to neuroinvasion and acid tolerance and the unique genetic characteristics in clinical strains of *E. anophelis* compared with mosquito strains may also provide insights on the ability of *E. anophelis* to adapt to different ecologic niches and cause neonatal infection through vertical transmission.

In conclusion, the genome data we obtained for these cases offered superior discriminatory power that supported appropriate infection control measures. The ability to distinguish different bacterial isolates often has critical implications on practical infection-control management, but different strains of the same bacterial species may not be distinguishable by their phenotypes because they reflect a tiny portion of the microbial genome. With better automation and lower costs, draft genome sequencing, which

offers a short turnaround time, may replace existing typing methods such as PFGE or multilocus sequence typing for outbreak investigations.

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Microbiota That Affect Risk for Shigellosis in Children in Low-Income Countries

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Pathogens in the gastrointestinal tract exist within a vast population of microbes. We examined associations between pathogens and composition of gut microbiota as they relate to *Shigella* spp./enteroinvasive *Escherichia coli* infection. We analyzed 3,035 stool specimens (1,735 nondiarrheal and 1,300 moderate-to-severe diarrheal) from the Global Enteric Multicenter Study for 9 enteropathogens. Diarrheal specimens had a higher number of enteropathogens (diarrheal mean 1.4, nondiarrheal mean 0.95; p<0.0001). Rotavirus showed a negative association with *Shigella* spp. in cases of diarrhea (odds ratio 0.31, 95% CI 0.17–0.55) and had a large combined effect on moderate-to-severe diarrhea (odds ratio 29, 95% CI 3.8–220). In 4 *Lactobacillus* taxa identified by 16S rRNA gene sequencing, the association between pathogen and disease was decreased, which is consistent with the possibility that *Lactobacillus* spp. are protective against *Shigella* spp.–induced diarrhea. Bacterial diversity of gut microbiota was associated with diarrhea status, not high levels of the *Shigella* spp. *ipaH* gene.

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Diarrheal disease contributes substantially to illness and death in children in low-income countries (*1*,*2*). Recent investigations of enteric illness have shown many cases with >1 pathogen identified (*3*–*5*). The paradigm of 1 pathogen and 1 disease has been questioned with the advent of microbiological and molecular detection methods that have lower limits of detection. Children in developing countries are exposed to an array of pathogenic organisms. Recent studies have shown a complex relationship between gut microbiota and diarrheal illness; children with severe illness tend to have a less diverse microbiota and a predominance of specific genera of organisms (*6*). Molecular-based approaches to pathogen detection enhance the ability to quantify the abundance of pathogens shed in the stool.

Two recent studies of children in low-income countries have highlighted the need for pathogen quantitation. Lindsay et al., using the Global Enteric Multicenter Study (GEMS) specimen collection, found that 80% of controls and 89% of case-patients had detectable levels of *Shigella* spp. (*7*). To identify which children had shigellosis, Lindsay et al. determined a quantitative threshold and, when applied, it identified twice as many cases compared with standard culture. Platts-Mills et al., in a study of populations with a high prevalence of malnutrition and enteric infections in Tanzania, compared samples taken before and during diarrheal episodes (*8*). They did not find an association between the presence of any pathogen and diarrhea for 15 pathogens studied (rotavirus, adenovirus, astrovirus, norovirus, sapovirus, *Cryptosporidium* spp., *Giardia lamblia*, *Campylobacter jejuni*, *Clostridium difficile*, *Salmonella* spp., Shiga-toxigenic *Escherichia coli*, *Shigella* spp./enteroinvasive *E. coli* [EIEC], enterotoxigenic *E. coli* [ETEC], typical enteropathogenic *E. coli* [tEPEC], and enteroaggregative *E. coli* [EAEC]). However, when they considered quantity of pathogen on a continuous scale, 3 organisms (rotavirus, astrovirus, and *Shigella* spp.) were associated with diarrhea.

In disease-endemic settings, detection of multiple enteropathogens in asymptomatic and symptomatic children is common (*4*,*9*). Samples from one third of patients with diarrhea in a hospital study in Kolkata, India, contained >1 pathogen. Negative associations were demonstrated between *Shigella* spp. and rotavirus and *Shigella* spp. and *Vibrio cholerae* (*3*). However, a limitation of this study was that it was conducted only in patients with diarrhea. Thus, differential comparisons could not be made between pathogen associations in diarrheal and nondiarrheal samples. A recent study by Taniuchi et al. reported the etiology of diarrheal episodes by using molecular methods in Bangladeshi children during their first year of life. They found that multiple enteropathogens were present by the first month of life in stool specimens from healthy children and from children with diarrhea (*4*). If multiple pathogens are present, they might interact to increase or decrease the probability of symptomatic infection. Bhavnani et al. reported synergistic effects in rotavirus–*G. lamblia* and rotavirus–*E. coli* infections, in which the presence of these co-occurring organisms increased the probability of disease (*10*).

The gut microbiota are composed of thousands of species that might play a role in the risk for diarrhea. Some *Lactobacillus* and *Veillonella* species are potentially protective against diarrhea or serve as markers of healthy gut microbiota (*11*–*13*). Probiotic activity has been associated with some *Lactobacillus* spp., bifidobacteria, *Veillonella* spp., *Streptococcus* spp., *Enterococcus* spp., nonpathogenic *E. coli*, and *Saccharomyces boulardii* (*13*). Randomized clinical trials have investigated the role of some *Lactobacillus* spp. in treating infectious diarrhea and identified that these organisms can provide a benefit in the treatment of acute, infectious, watery diarrhea in infants and young children (*12*). In this study, we examined relationships between *Shigella* spp./EIEC, microbiota, and diarrhea by using 16S rRNA marker gene surveys of stool specimens from a large international study of diarrhea in children <5 years of age (*9*).

Methods

Study Design and Participants

We used stool specimens collected from children <5 years of age who participated in a matched case–control study of moderate-to-severe diarrhea sponsored by the GEMS consortium (*14*,*15*). In brief, the GEMS was a prospective case–control study of infants and young children at 7 sites in sub-Saharan Africa and southern Asia. Case-patients with moderate-to-severe diarrhea were enrolled when they came to a health clinic. Moderate-to-severe diarrhea eligibility criteria included dehydration (sunken eyes, loss of normal skin turgor, or a decision to initiate intravenous hydration), the presence of blood in the stool (dysentery), or a clinical decision to hospitalize the child. In the GEMS, matching controls (for sex, age, and community) were sampled from a demographic surveillance database of the area and included if they reported no diarrhea within the previous 7 days. For this study, 4 of the 7 GEMS sites (The Gambia, Mali, Kenya, and Bangladesh) elected to participate in further molecular characterization of their samples. When samples were sent for analysis, it was not required that their matched specimen be included, which resulted in disruption of matches. Including only samples with the complete matched set would have limited our sample size. Therefore, we included age and location as covariates in our analysis but did not analyze samples as matched sets.

All specimens for this study were collected during December 2007–December 2009. One specimen was collected for each child at the time of enrollment. The Institutional Review Boards at all participating institutions reviewed and approved the protocol.

Specimen Collection

Stool specimens were handled according to the GEMS protocol (*16*). DNA was isolated from frozen stool specimens by using a bead beater with 3-mm diameter solid glass beads (Sigma-Aldrich, St. Louis, MO, USA) and, subsequently, with 0.1-mm zirconium beads (BioSpec Products, Inc., Bartlesville, OK, USA) to disrupt cells. The cell slurry was then centrifuged at 16,000 × *g* for 1 min, and the supernatant was processed by using QIAamp DNA Stool Extraction Kit (QIAGEN, Valencia, CA, USA). Extracted DNA was precipitated with ethanol and shipped to the United States.

Detection of EAEC, tEPEC, ETEC, Rotavirus, Norovirus, *G. lamblia*, and *Cryptosporidium* spp.

Diagnostic microbiological methods for rotavirus, norovirus, *G. lamblia*, *Cryptosporidium* spp., and diarrheagenic *E. coli* (EAEC, tEPEC, and ETEC) were conducted at each site as described by Panchalingham et al. (*16*). In brief, *E. coli* isolates were selected from MacConkey agar plates and tested by using motility indole ornithine medium. EAEC, tEPEC, and ETEC pathotypes were identified by using PCRs for known virulence determinants of each pathogen (ETEC: heat-labile and heat-stable enterotoxins; tEPEC: intimin [*eae*] and bundle-forming pilus; and EAEC: *aatA* and *aaiC* genes).

Rotavirus was detected by using the ProSpecT ELISA Rotavirus Kit (Oxoid, Basingstoke, UK). Norovirus genogroups GI and GII were detected by using a multiplex PCR specific for synthesized complementary DNA after viral RNA extraction and reverse transcription. *G. lamblia* and *Cryptosporidium* spp. were detected by using commercially available immunoassays (TechLab, Blacksburg, VA, USA) following the manufacturer's protocols. Although the GEMS study tested for other enteric pathogens, our

analysis included only pathogens that were present in ≥10 specimens. This lower limit was chosen after examination of the distribution of positive samples for each pathogen and to avoid statistical testing with small sample sizes.

Quantitative PCR for Detection of *ipaH* Gene in *Shigella* spp. and EIEC

Each stool DNA specimen was tested by using a quantitative PCR (qPCR) for *Shigella*/EIEC that included the 7500/700 Fast Real-Time PCR System, software V2·0·5, and SYBR green–based fluorescent dye (Applied Biosystems, Foster City, CA, USA). Details on primer design, PCR conditions, and standard curve analysis have been reported elsewhere (*4*,*7*,*17*). *Shigella*/EIEC was identified by using primers specific for the *ipaH* gene (*7*). We used a cutoff of 14,000 *ipaH* gene copies to distinguish children shedding low levels of *Shigella* spp. from those shedding high levels of *Shigella* spp. in their stools. The threshold was established by constructing receiver-operating characteristic curves to determine sensitivity and specificity of incremental increases in levels of *ipaH* compared with disease status and set on the basis of the point that maximized sensitivity and specificity (*7*). Stool specimens with high and low levels of *ipaH* indicate the relative amount of *Shigella* spp. detected.

16S rRNA Gene Sequencing and Analysis and Identification of *C. jejuni*

DNA was amplified by using universal primers specific for the V1–V3 region of the 16S rRNA gene in bacteria (518R [5′-CAATTACCGCGGCTGCTGG-3′] and 27F [5′-AGAGTTTGATCCTGGCTCAG-3′]). Individual reads were filtered for quality by using custom in-house scripts that removed low-quality sequences as described (*6*). Remaining high-quality sequences were separated into sample-specific sets according to barcodes. Conservative operational taxonomic units (OTUs) were clustered by using DNACLUST with parameters (–r 1 and 99% identity clusters) to ensure that the definition of an OTU was consistent across all samples (*18*). For taxonomic identification, a representative sequence from each OTU was aligned to the Ribosomal Database (RDP) (rdp.cme.msu.edu, release 10.4) by using BLASTn (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with long word length (–W 100) to detect only nearly identical sequences (*19*). Sequences without a nearly identical match to the RDP (>100-bp perfect match and >97% identity, as defined by BLAST) were marked as being unassigned and assigned a unique OTU identifier. If a sample contained taxa classified as *C. jejuni*, we identified this sample as positive for *C. jejuni*.

Statistical Analysis

Associations between high levels of *ipaH* and each additional pathogen, stratified by diarrheal status, were assessed for children with moderate or severe diarrhea and for controls by using separate logistic regression models with high levels of *ipaH* as the dependent variable. Logistic regression with moderate-to-severe diarrhea as the outcome of interest was then conducted to test for the interaction between high levels of *ipaH* and either 1) pathogenic microorganisms tested for by the GEMS or 2) species identified by 16S rRNA gene sequencing. All models were adjusted for potential confounding caused by location and age with categorical location and age terms in the model.

Statistical modeling was used to examine whether microbes interact to effect diarrhea risk. To assess whether the risk caused by having *Shigella* spp. and an additional microbe differed from the product of the risks caused by having each microbe separately (i.e., multiplicative interaction), we used a logistic regression model with an interaction term containing the level of *ipaH* and the additional microbe of interest (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/2/14-0795-Techapp1.pdf). To assess whether the excess risk of having *ipaH* and an additional microbe differed from the sum of the excess risk of having each separately (i.e., additive interaction), we estimated the relative excess risk caused by the interaction (RERI, also called the interaction contrast ratio [ICC]) (*20*–*22*). An RERI = 0 indicates no additive interaction, an RERI>0 suggests positive additive interaction, and an RERI<0 suggests negative additive interaction. Ninety-five percent CIs were estimated by using the Hosmer-Lemeshow procedure (*23*,*24*). All statistical analysis was performed by using SAS version 9·2 (SAS Institute, Cary, NC, USA) and R 2.15 (*25*), and p values <0.05 were considered significant.

Results

A total of 3,035 (1,735 nondiarrheal and 1,300 diarrheal) stool specimens from children <5 years of age from The Gambia, Mali, Kenya, and Bangladesh were examined for *Shigella* spp. by identification of *ipaH* by qPCR; for *G. lamblia*, *Cryptosporidium* spp., and rotavirus by ELISA; for norovirus by PCR; for EAEC, ETEC, or tEPEC by culture and subsequent PCR; and for *C. jejuni* by 16S rRNA gene sequencing. Characteristics of samples are shown in Table 1. Diarrheal samples had a significantly higher number of pathogens (diarrheal mean 1.4, nondiarrheal mean = 0.95; p<0.05).

Associations and Interaction Effects of Co-occurring Pathogens

In 69% (278/404) of samples with high levels of *ipaH* (71% of diarrheal samples with high levels of *ipaH* and 64% nondiarrheal samples with high levels of *ipaH*), we identified an additional pathogen. In samples with low levels of *ipaH*, we identified an additional pathogen in 69% (1,815/2,631; 80% of diarrheal samples with low levels of *ipaH* and 62%

Table 1. Characteristics and pathogen abundance in children with cases of moderate-to-severe diarrhea and controls in low-income countries*

Characteristic	Cases, n = 1,300	Controls, n = 1,735	p value	Total, n = 3,035
Male sex	727 (56)	965 (56)	0.8677	1,692 (56)
Age, mo, mean (SD)	16.7 (12)	17.8 (12)	0.0073	17.39 (12)
0–5	156 (12)	177 (10)	0.1167	333 (11)
6–11	413 (32)	465 (27)	0.0028	878 (29)
12–23	431 (33)	617 (35)	0.1674	1,048 (35)
24–35	185 (14)	311 (18)	0.0064	496 (16)
36–59	115 (9)	165 (10)	0.5318	280 (9)
Country	NA	NA	NA	NA
The Gambia	356 (27)	408 (23)	0.0151	764 (25)
Mali	103 (8)	114 (7)	0.1524	217 (7)
Kenya	636 (49)	779 (45)	0.0279	1,415 (47)
Bangladesh	205 (16)	434 (25)	<0.0001	639 (21)
ipaH gene copies ≥14,000	277 (22)	127 (7)	<0.0001	404 (13)
Rotavirus	183 (14)	41 (2)	<0.0001	224 (7)
Norovirus genogroups GI or GII	127 (10)	141 (8)	0.1146	268 (9)
Giardia lamblia	230 (18)	373 (22)	0.0093	603 (20)
Cryptosporidium spp.	142 (11)	87 (5)	<0.0001	229 (7)
tEPEC	106 (8)	104 (6)	0.0203	210 (7)
EAEC	253 (19)	347 (20)	0.7124	600 (20)
ETEC	181 (14)	148 (8)	<0.0001	329 (11)
Camplylobacter jejuni	341 (26)	264 (15)	<0.0001	605 (20)
Total pathogens, mean (SD)	1.4 (0.9)	0.9 (0.9)	<0.0001	1.2 (0.9)

*Values are no. (%) children unless otherwise indicated. NA, not applicable; tEPEC, typical enteropathogenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*.

of nondiarrheal samples with low levels of *ipaH*). After adjusting for age and location, rotavirus exhibited a negative association with high levels of *ipaH* in case-patients (odds ratio [OR] 0.31, 95% CI 0.17–0.55). In case-patients and controls, no other pathogen showed an association (Figure 1; online Technical Appendix Table 1).

Of the 8 pathogens tested, none showed any interaction effects. However, rotavirus was negatively associated with high levels of *ipaH* (Table 2), and only 15 samples that had high levels of *ipaH* were positive for rotavirus; 14 of these 15 samples were diarrheal samples. The presence of rotavirus and high levels of *ipaH* resulted in an OR of 29 (95% CI 3.8–220) for diarrheal risk. Although this point estimate was much higher than the expected additive effect of 11, this result and results of tests for interaction were not statistically significant (Table 3).

Interaction Effects of *Lactobacillus* and *Veillonella* Taxa

A total of 61 *Lactobacillus* and *Veillonella* taxa were identified by 16S rRNA gene sequencing. Analyses were limited to 31 taxa that co-occurred in samples with high levels of *ipaH* ≥10 times. We tested for interaction between high levels of *ipaH* and the identified 16S rRNA gene taxon by using a logistic regression model adjusted for age and location, including an interaction term between level of *ipaH* and taxon presence (online Technical Appendix Table 2). Five taxa showed significant negative multiplicative interactions identified by an interaction term with $p \leq 0{\cdot}05$. Of these taxa, *Lactobacillus ruminis* (RERI –1.92, 95% CI –3.36 to 0.47), *Lactobacillus* DJF-RP24 (RERI –2.44, [95% CI –3.93 to 0.95), *Lactobacillus* KLDS 1.0718 (RERI –1.93, 95% CI –3.56 to

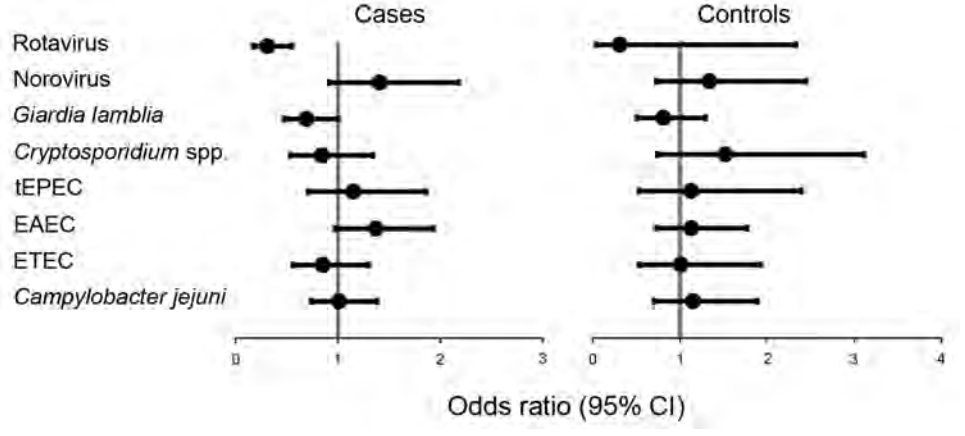

Figure 1. Association of co-occurring pathogens with high levels of *ipaH* gene of *Shigella* spp. in stool specimens of children with diarrhea (cases) and children without diarrhea (controls) in low-income countries. Dark circles indicate means, and error bars indicate 95% CIs. tEPEC, typical enteropathogenic *Escherichia coli*; EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*.

Table 2. Interaction between level of *Shigella* spp./*EIEC ipaH* gene and rotavirus and association with moderate-to-severe diarrhea in children in low-income countries*

Rotavirus status†	Low level of *ipaH* gene		High level of *ipaH* gene		OR (95% CI) for *ipaH* gene within strata of rotavirus status
	No. with MSD/ no. without MSD	OR (95% CI)	No. with MSD/ no. without MSD	OR (95% CI)	
Negative	854/1,568	1.00 (reference)	263/126	4.01 (3.18–5.05)	4.01 (3.18–5.05)
Positive	169/40	7.56 (5.29–10.80)	14/1	28.85 (3.77–220)	3.82 (0.48–30.00)
OR (95% CI) for rotavirus within strata of *ipaH* gene status	NA	7.55 (5.29–10.80)	NA	7.20 (0.93–55.57)	Expected additive = 10.57

*EIEC, enteroinvasive *Escherichia coli*; MSD, moderate-to-severe diarrhea; OR, odds ratio; NA, not applicable.
†Model is unconditional and adjusted for age and location.

0.29), and *Lactobacillus* TSK G32.2 (RERI -2.69, 95% CI -4.55 to 0.84]) showed additive interactions (Table 3). The combined effect of high levels of *ipaH* in the presence of *L. ruminis, Lactobacillus* DJF RP24, *Lactobacillus* KLDS 1.0718, or *Lactobacillus* TSK G32.2 was lower than expected (Figure 2; online Technical Appendix Table 3), which suggested an antagonistic interaction or a decreased association with diarrhea when specific *Lactobacillus* taxa were present than versus when they were absent. When we tested the 4 *Lactobacillus* taxa against 8 additional pathogens; no additive interaction was observed (online Technical Appendix Table 4).

16S rRNA Gene–based Bacterial Community Profiles

The proportional abundance of the 9 most common genera identified by 16S rRNA marker gene sequencing differed among stool specimens with high and low levels of *ipaH* and among specimens from children with and without diarrhea (Figure 3). Overall composition of diarrheal stool specimens had an increased relative proportional abundance of facultative anaerobes when compared with composition of nondiarrheal stool specimens, even when diarrheal stool specimens had low levels of *ipaH* (mean diarrheal specimens 0.47, mean nondiarrheal specimens 0.22; p<0.0001). Overall, diarrheal stool specimens with high levels of *ipaH* had the lowest proportion abundance of *Prevotella* spp. (0.11), diarrheal stool samples with low levels of *ipaH* had the second lowest proportional abundance (0.15), and nondiarrheal stool specimens with high and low levels of *ipaH* had similar proportion abundances for *Prevotella* spp. (0.25 and 0.24, respectively; p = 0.63).

Shigella spp. identified by the *ipaH* qPCR were a subset of the genera *Escherichia/Shigella* identified by 16S rRNA gene sequencing, but could not be distinguished from commensal strains. Diarrheal specimens had a larger proportion of members of the genera *Escherichia/Shigella* (p<0.0001) than nondiarrheal specimens, regardless of levels of *ipaH*. However, diarrheal stool specimens with high levels of *ipaH* had a significantly higher proportion of *Escherichia/Shigella* sequences (31%; p<0.0001) than did diarrheal stool specimens with low levels of *ipaH*.

Members of the genus *Veillonella* was found equally in diarrheal and nondiarrheal stool specimens. Streptococci

Table 3. Measures of additive and multiplicative interactions of co-occurrence of *Shigella* spp. and another pathogen or *Lactobacillus* spp. in children in low-income countries*

Pathogen or organism	OR_{10}† (95% CI)	OR_{01}‡ (95% CI)	OR_{11}§ (95% CI)	RERI (95% CI)	Additive p value	Multiplicative p value
C. jejuni	3.84 (2.98–4.95)	1.97 (1.62–2.40)	5.55 (3.67–9.15)	0.79 (–2.15 to 3.73)	0.62	0.29
ETEC	3.77 (2.97–4.80)	1.69 (1.32–2.18)	4.65 (2.32–9.30)	0.15 (–3.20 to 3.52)	0.92	0.41
tEPEC	3.69 (2.91–4.69)	1.36 (1.02–1.83)	4.09 (2.01–8.33)	0.03 (–2.99 to 3.06)	0.99	0.61
Cryptosporidium spp.	3.79 (2.99–4.81)	2.40 (1.78–3.24)	4.95 (2.28–10.75)	–0.27 (–4.27 to 3.73)	0.91	0.16
Rotavirus	4.01 (3.18–5.05)	7.56 (5.29–10.80)	28.85 (3.77–220)	18.29 (–40.32 to 76.91)	0.54	0.96
Norovirus genogroups GI or GII	3.65 (2.87–4.64)	1.14 (0.86–1.51)	4.00 (2.08–7.71)	0.21 (–2.56 to.98)	0.88	0.91
EAEC	3.59 (2.78–4.63)	0.87 (0.71–1.07)	3.29 (2.06–5.26)	–0.17 (–1.92 to 1.58)	0.85	0.86
Giardia lamblia	3.65 (2.84–4.69)	0.83 (0.68–1.02)	2.70 (1.63–4.49)	–0.78 (–2.83 to 0.83)	0.35	0.70
Lactobacillus taxon						
KLDS 1.0718	**4.10 (3.19–5.26)**	**1.25 (1.01–1.55)**	**2.42 (1.41–4.13)**	**–1.93 (–3.56 to 0.29)**	**0.02**	**0.02**
L. salivarius	4.12 (3.20–5.31)	1.77 (1.45–2.16)	3.88 (2.35–6.38)	–1.02 (–3.18 to 1.14)	0.36	**0.03**
L. ruminis	**4.30 (3.19–5.79)**	**0.82 (0.69–0.96)**	**2.20 (1.55–3.13)**	**–1.92 (–3.36 to 0.47)**	**0.01**	**0.05**
DJF RP24	**4.59 (3.41–6.16)**	**0.85 (0.72–0.99)**	**1.99 (1.39–2.85)**	**–2.44 (–3.93 to 0.95)**	**0.001**	**0.01**
TSK G32–2	**3.89 (3.08–4.92)**	**1.40 (1.07–1.85)**	**1.60 (0.59–4.30)**	**–2.69 (–4.55 to 0.84)**	**0.004**	**0.02**

*OR, odds ratio; RERI, relative excess risk caused by the interaction; ETEC, enterotoxigenic *Escherichia coli*; tEPEC, typical enteropathogenic *E. coli*; EAEC, enteroaggregative *E. coli*. Boldface indicates significance (p≤0.05). Under an additive interaction, $OR_{11} - OR_{10} - OR_{01} + 1 = 0$. Under a multiplicative interaction, $OR_{11} = OR_{10}OR_{01}$.
†OR caused by having a high level of the *ipaH* gene alone (in the absence of the other pathogen or *Lactobacillus* taxon).
‡OR caused by having the other pathogen or *Lactobacillus* taxon alone (in the absence of a high level of the *ipaH* gene).
§OR caused by having a high level of the *ipaH* gene and another pathogen or *Lactobacillus* taxon (relative to having neither).

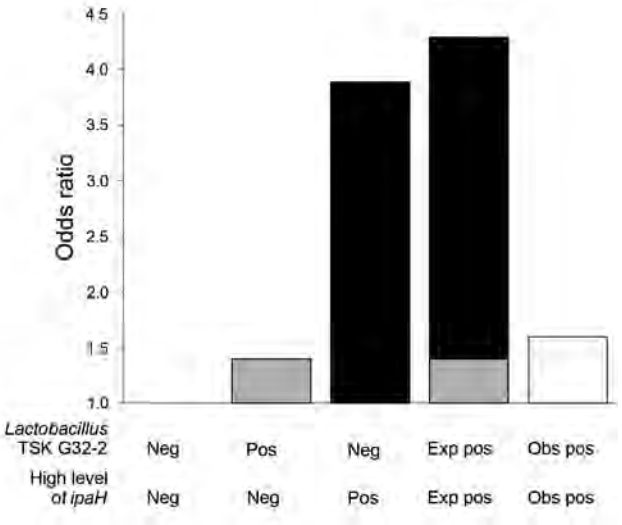

Figure 2. Departure from additivity between level of *ipaH* gene and presence of *Lactobacillus* taxon TSK G32-2 on odds of moderate-to-severe diarrhea in children in low-income countries. The reference group is TSK G32-2 negative, low level of *ipaH*. The observed combined joint effect of a high level of the *ipaH* gene and TSK G32-2 was lower than the expected additive effect. Gray bars indicate effect of TSK G32-2; black bars indicate effect of high levels of *ipaH*; and white bar indicates observed joint effect of TSK G32-2 and high levels of *ipaH*. Neg, negative; Pos, positive; Exp, expected; Obs, observed.

were found more often in diarrheal stool specimens, regardless of levels of *ipaH*. There was no association between Shannon diversity indices and levels of *ipaH* (p = 0.95), although diversity was significantly associated with age, location, and moderate-to-severe diarrhea (p<0.0001, p = 0.004, and p<0.0001, respectively) (Figure 4).

Discussion

In this study, we used qPCR and 16S rRNA gene sequencing to identify interactions between *Shigella*/EIEC and co-occurring enteric pathogens or microbes within the gut microbiota in children with moderate-to-severe diarrhea. We explored these interactions in stool specimens obtained from children with and without diarrhea who participated in the international GEMS study. We used detection of the *ipaH* gene as an indicator of *Shigella*/EIEC infection because this molecular method is highly sensitive and specific (*4*,*26*,*27*). Use of quantitative identification methods, rather than colonization (*7*,*8*), is advantageous for identifying true disease associations. We found that 69% (1,815/2,631) of stool samples with high levels of *ipaH* had a co-occurring pathogen.

This study confirms the findings of the negative association between rotavirus and *Shigella* spp. in polymicrobial infections in diarrheal patients in India and The Gambia (*Shigella* spp./rotavirus: OR 0.36, 95% CI 0.14–0.92) (*3*,*28*). Although we identified correlations between rotavirus and *Shigella* spp. as determined by levels of *ipaH*, no pathogen showed an antagonistic or synergistic interaction on the odds of moderate-to-severe diarrheal illness. Although all but 1 of the rotavirus-positive samples that had high levels of *ipaH* were associated with diarrhea cases, the negative association between these pathogens gave us limited sample size to assess a possible synergistic effect. A synergistic effect between high levels of *ipaH* and rotavirus (RERI/ICC 9.9, 95% CI 2.6–28.4) was previously observed in Ecuador, and it was concluded that pathogenic potential appears to be enhanced during co-infection (*10*). Our RERI/ICC was 18, but the effect was not significant, possibly because of small sample size.

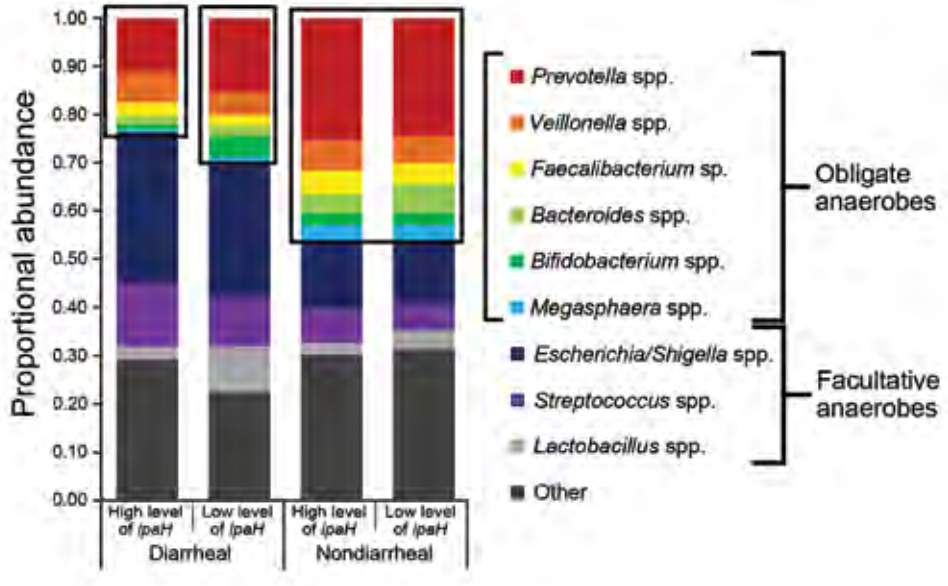

Figure 3. Overall 16S rRNA gene–based bacterial community profiles (proportional abundance) of diarrheal samples with high levels of *ipaH* gene (n = 277), diarrheal samples with low levels of *ipaH* gene (n = 1,023), nondiarrheal samples with high levels of *ipaH* gene (n = 127), and nondiarrheal samples with low levels of *ipaH* gene (n = 1,608) from children in low-income countries. Other indicates sequences that were not identified as 1 of the 9 most abundant taxa or did not have good (>100 bp exact match, >97% identity) matches with isolate sequences from the Ribosomal Database Project (*19*).

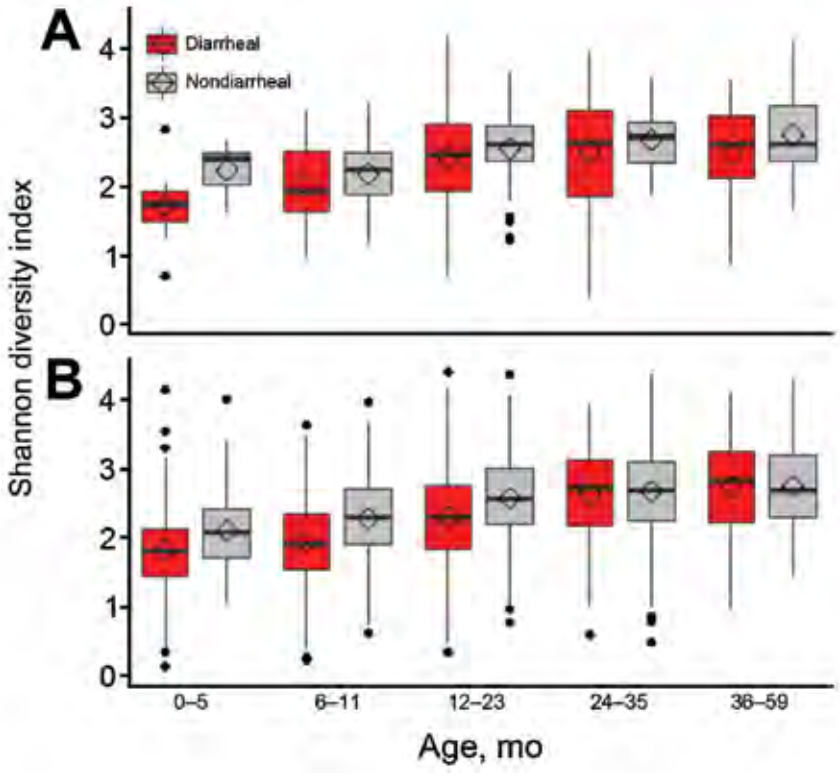

Figure 4. Shannon diversity index for diarrheal and nondiarrheal samples with high (A) and low (B) levels of *Shigella* spp. *ipaH* gene stratified by age group for children in low-income countries. Box and whisker plot indicates distribution of diversity index for each group. The upper whisker extends from the 75th percentile to the highest value that is ≤1.5× the interquartile range (IQR) of the hinge (upper end) or the distance between the first and third quartiles. The lower whisker extends from the hinge (lower end) to the lowest values 1.5× the IQR of the hinge. Diamonds indicate means and horizontal lines indicate medians. Points outside the ends of the whiskers are outliers beyond 1.5× the IQR of the hinge.

The GEMS was designed as a matched case–control study of thousands of stool samples from persons matched by age, sex, community, and time. A possible limitation of our methods is that we did not use matched pairs but rather adjusted for country-level location and age by using statistical modeling. This method was used because of the large number of broken matched pairs. Thus, inclusion of all samples greatly increased our sample size. Generally, the lack of an appropriate matched analysis biases the effect on estimates toward the null. However, studies have shown that including samples with missing data and adjusting by using confounding terms is valuable, particularly with a larger sample size (*29*,*30*). We adjusted for location at the country level, and the GEMS matched case-patients at the community level, which could fail to adequately address bias, particularly when one considers that the distribution of case-patients and controls differed at multiple sites and that *Shigella* spp. are more common in Bangladesh. An additional limitation of our study design is that, given a cross-sectional study sample set, we were unable to identify temporal associations and to attribute cause and effect. Furthermore, associations between pathogens and taxa may be explained by seasonality, and further work should be conducted to investigate this as a possible explanation. Finally, the 16S rRNA gene-sequencing method used to identify taxa of interest is limited because our identification was only as precise as the RDP attributed taxonomy. Thus, further characterization and species-specific identification are warranted in uncultured bacteria such as *Lactobacillus* KLDS 1.0718.

A previous study showed cross-sectional differences in stool microbiota of children with diarrhea compared with children without diarrhea in low-income countries (*6*). Our study showed that the composition of microbiota is more closely associated with diarrheal status than with co-infection by *Shigella* spp./EIEC as measured by high levels of *ipaH*. Microbiota in stools of children without diarrhea but who had high levels of *ipaH* (i.e., were colonized with *Shigella* spp./EIEC) were more similar to microbiota of healthy children with low levels of *ipaH* than microbiota in samples from children with diarrhea and high levels of *ipaH*.

In low-income countries, infection/colonization with pathogens occurs commonly in persons without diarrhea. Our study found little evidence of interaction between *Shigella* spp. and co-occurring pathogens. Although the cross-sectional study design precludes strong statements of cause and effect, our data are consistent with the possibility that some *Lactobacillus* taxa naturally occurring in the gut and are protective against *Shigella* spp.–induced diarrhea. Future studies should continue to consider the effects of co-occurring species.

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B.L., J.P.N., and O.C.S. developed the hypotheses; M.M.L., K.K., and J.P.N. led the Global Enteric Multicenter Study; B.L., J.O., M.A.H., M.A., B.T., R.O., A.S.G.F., S.K.D., U.N.I., M.A., D. Sanogo, D. Saha, S.S., T.H.F., D.N., and S.P. collected data and developed and maintain databases; A.W.W., J.N.P., J.P., M.P., and O.C.S. developed and performed 16S rRNA gene sequencing, assembly, and taxonomic identification; S.L., B.L., and S.P. performed laboratory protocols; and B.L., L.S.M., L.H., and H.S. performed analyses.

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At the time of this study, Dr. Lindsay was a research analyst and doctoral student at the University of Maryland, Baltimore, Maryland. Her research interests are identification and quantitation of *Shigella* spp. and how this pathogen interacts with human gut microbiota.

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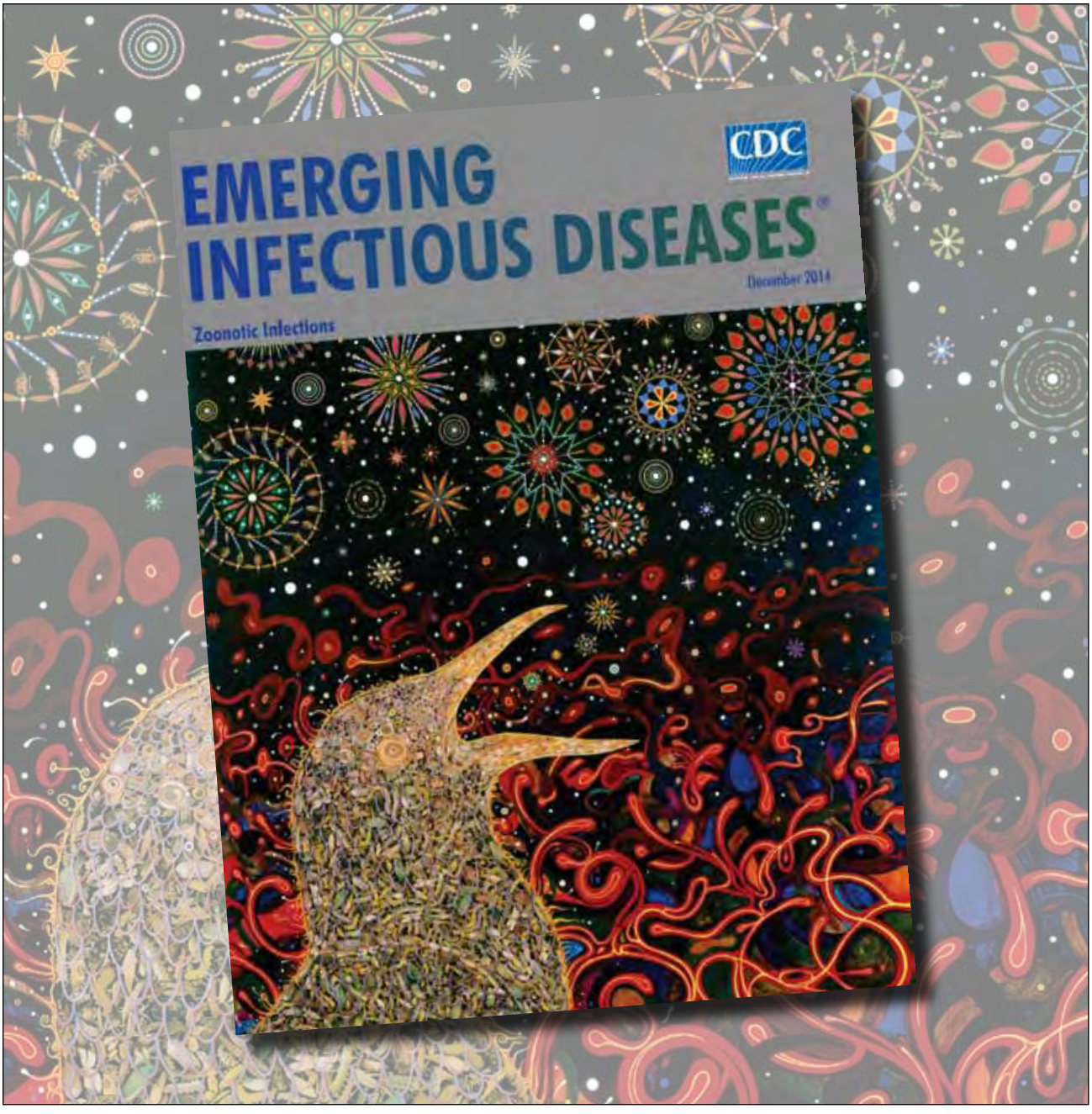

Optimizing Distribution of Pandemic Influenza Antiviral Drugs

Bismark Singh, Hsin-Chan Huang, David P. Morton, Gregory P. Johnson, Alexander Gutfraind, Alison P. Galvani, Bruce Clements, Lauren A. Meyers

We provide a data-driven method for optimizing pharmacy-based distribution of antiviral drugs during an influenza pandemic in terms of overall access for a target population and apply it to the state of Texas, USA. We found that during the 2009 influenza pandemic, the Texas Department of State Health Services achieved an estimated statewide access of 88% (proportion of population willing to travel to the nearest dispensing point). However, access reached only 34.5% of US postal code (ZIP code) areas containing <1,000 underinsured persons. Optimized distribution networks increased expected access to 91% overall and 60% in hard-to-reach regions, and 2 or 3 major pharmacy chains achieved near maximal coverage in well-populated areas. Independent pharmacies were essential for reaching ZIP code areas containing <1,000 underinsured persons. This model was developed during a collaboration between academic researchers and public health officials and is available as a decision support tool for Texas Department of State Health Services at a Web-based interface.

Influenza pandemics occur when novel strains of the influenza virus emerge in human populations and spread worldwide (*1*). There were 3 influenza pandemics in the 20th century (1918, 1957, and 1968), and 1 has occurred so far in the 21st century (2009). The 1918 Spanish flu pandemic was far more severe than the others, causing an estimated 50 million deaths globally (*2*). In contrast, the 2009 pandemic had an estimated death toll of 284,000 (*3*). Experts conjecture that the risk for new pandemics will increase in the coming decades (*4*), and several emerging threats are already under surveillance. A highly pathogenic avian influenza A (H5N1) virus has occasionally been infecting humans in Asia, Africa, and Europe since 1997 (*5*); the first human case in North America was reported in January 2014 (*6*). Since March 2013, China has been trying to contain an ongoing outbreak of a highly pathogenic avian influenza (H7N9) virus (*7*,*8*).

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The primary control measures for pandemic influenza are antiviral medications and vaccines (*9*), as well as nonpharmaceutical interventions, such as social distancing measures, school closures, and hygienic precautions (*10*). Although the efficacy of influenza vaccines depends on factors such as patient age and virus type/subtype (*11*), these vaccines are arguably the best intervention strategy (*1*). However, because development and deployment of effective vaccines for a new influenza virus may take several months (*12*), antiviral drugs and nonpharmaceutical interventions are particularly critical for early pandemic control.

Antiviral drugs are believed to reduce disease severity and duration of infectiousness in individual patients, if taken sufficiently early (*9*), and to protect contacts of infected persons, if taken prophylactically (*13*–*15*). Some studies have suggested that aggressive treatment policies can effectively mitigate local transmission (*16*,*17*). In preparation for future influenza pandemics, the US Department of Health and Human Services therefore maintains a large Strategic National Stockpile (SNS) of antiviral drugs (*18*), and most states include SNS antiviral drugs as a major component of their pandemic response plans (*19*–*22*).

After detection of the new influenza A(H1N1)pdm09 virus in April 2009, the US government declared a public health emergency, and the World Health Organization declared a global influenza pandemic. Vaccines became widely available after 6 months of sustained transmission (*23*). In the early weeks of the pandemic, the US Department of Health and Human Services distributed 11 million courses of federally held SNS antiviral drugs to states (*24*) and issued a series of guidelines for implementing antiviral drug and nonpharmaceutical interventions. During the pandemic, many states sought to work in cooperation with retail pharmacies and independent drug stores to assist in dispensing their shares of the SNS and state caches (*19*–*22*). The Texas Department of State Health Services (DSHS) enlisted the help of several major pharmacy chains and independent retail pharmacies to dispense >200,000 antiviral drug courses from the SNS and state cache (*25*). A report analyzing the pandemic response indicated that additional planning is required to ensure that persons residing in counties in Texas lacking pharmacies can obtain antiviral drugs when needed (*26*).

We propose a method for optimizing the location of dispensing points for antiviral drugs within a state. Several state pandemic response plans include the following goals: using commercial pharmacies as antiviral drug-dispensing partners to limit strain on hospitals; reaching a population of broad demographics, including underinsured populations; and improving convenience. These states include Virginia (*19*), Louisiana (*20*), Florida (*21*), Tennessee (*22*), and Texas (*25*). These plans further specify that the choice of participating chains will depend on their location and local demographics, and they include strategies for reaching the underinsured population at minimal or no cost.

Working with Texas DSHS, we developed a data-driven facility-location model for designing commercial pharmacy antiviral drug distribution networks that maximizes access to underinsured populations, when access is based on a willingness-to-travel model estimated from National Household Travel Survey data (*27*). We describe the model that is now available to the Texas DSHS as a Web-based decision-support tool for future pandemics (*28*), and we use it to evaluate and optimize the commercial pharmacy distribution network established in Texas during the 2009 influenza pandemic.

Methods

Data

Texas has 1,939 US postal code (ZIP code) areas in 254 counties; 1,023 of these ZIP code areas contain ≥1 pharmacy (Table 1). We obtained the addresses of all community and clinic pharmacies with active licenses listed by the Texas Pharmacy Board (*29*). The largest chains (present in the most ZIP code areas) in Texas are Brookshire, Costco, CVS, HEB, Kmart, Kroger, Randalls, Sam's Club, Target, Tom Thumb, United, Walgreens, and Walmart. Other pharmacies, independent or small chain, are listed as independents. The Texas DSHS provided the list of pharmacies selected to dispense antiviral drugs to underinsured populations during the 2009 influenza pandemic; these pharmacies were in 723 ZIP code areas. To approximate the size of the uninsured and underinsured population in each ZIP code area (direct statistics were not available), we used the number of persons in households with an annual income <$20,000 (http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf).

Our optimization model uses a geographic resolution of ZIP code areas based on ZIP code tabulation areas (ZCTAs) (*30*). ZCTAs differ slightly from US Postal Service ZIP code areas and may include ≥1 US Postal Service ZIP code area. We mapped each pharmacy and residential ZIP code area to its corresponding ZCTA (*31*), and, for simplicity, we refer to these as ZIP code areas.

Willingness-to-Travel Model

We used National Household Travel Survey (NHTS) data for 2009 (*27*) to estimate the distances persons are willing to travel in Texas to obtain antiviral drugs sufficient for a course of treatment during an influenza pandemic (model described below). We created a willingness-to-travel model, which follows an exponentially decaying distribution, by fitting the model to national-scale NHTS data for privately operated vehicle travel (*27*) (Figure 1). This included ≈330,000 person trips (83% of all person trips in the database), totaling 3.3 million miles, including ≈30,000 person trips originating in Texas. We made the simplifying assumption that health care seeking behavior in Texas during an influenza pandemic will resemble national willingness to travel by privately operated vehicle for work, school, family, and social reasons. Although there are probably major differences in these estimates, we believe that this model conservatively underestimates actual accessibility of pharmacies during a pandemic.

Using a least-squares fit, we obtained the following model (Equation 1):

$$\hat{P}(d) = \begin{cases} \exp(-0.109 d^{1.184}) & \text{if } d < 5 \text{ (miles)} \\ 1.479 \exp(-0.4255 d^{0.6025}) & \text{if } d \geq 5 \end{cases}$$

in which the $\hat{P}(d)$ term is the fraction of the target population willing to travel at least d miles. As the required travel distance increases, the fraction of the population willing to travel distance d decreases. We used a piecewise model that allows for different coefficients below and above a distance threshold of 5 miles to enable urban and rural populations to exhibit different willingness-to-travel patterns (http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf).

To estimate travel patterns for the underinsured population, we considered NHTS data for households with incomes <$20,000 (http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf) and found that the travel patterns for this group are given by Equation 2:

$$\hat{P}_{at\_risk}(d) = \begin{cases} \exp(-0.1022 d^{1.21}) & \text{if } d < 5 \text{ (miles)} \\ 1.529 \exp(-0.433 d^{0.60}) & \text{if } d \geq 5 \end{cases}$$

The estimated willingness-to-travel for the underinsured population is slightly greater (<1%) than that for the entire population. The adjusted R^2 values for each model exceed 0.99.

Optimization Model

The optimization model we used identifies ZIP code areas for pharmacy-based distribution of SNS and state-cache antiviral drugs to maximize access in the target population

Table 1. Number of ZIP code areas containing ≥1 pharmacy of each chain and geographic overlap with Walgreens, which is present in the most ZIP code areas, Texas, USA*

Pharmacy name	No. ZIP code areas	Overlap with Walgreens, %
Walgreens	490	100.0
CVS/Pharmacy	422	75.1
Walmart	372	68.8
HEB	189	77.8
Kroger	167	86.8
Target	127	86.6
Brookshire	118	28.0
Sam's Club	74	89.2
Tom Thumb	51	74.5
Randalls	42	90.5
United	40	50.0
Costco	20	80.0
Kmart	16	81.3
Independents	893	48.0

*ZIP code areas, US postal code areas.

(either underinsured or entire population). It is a facility-location type model (*32,33*) with an objective function defined in terms of the expected number of persons willing to obtain antiviral drugs from the nearest dispensing point. We estimated this quantity by using our willingness-to-travel model for the distance between the home ZIP code centroid and pharmacy ZIP code centroid. For the distance to a pharmacy within the home ZIP code area, we used a correction factor based on the size of the ZIP code area (http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf).

The optimization model takes as input the total number of ZIP code areas to be included in the distribution network (*b*). The model does not account for the number of available antiviral drug doses, the number to be shipped

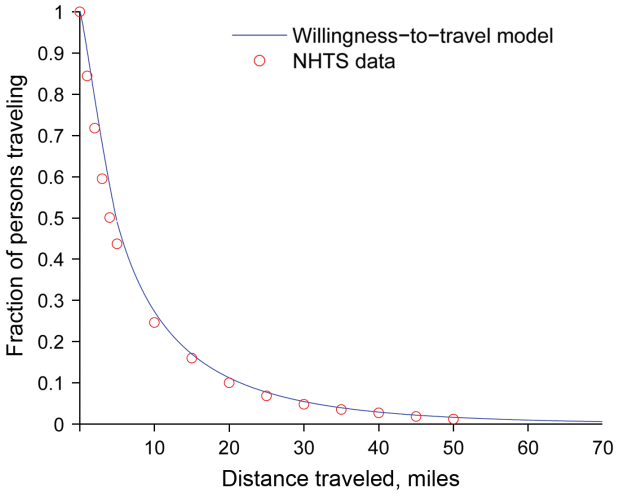

Figure 1. Willingness-to-travel curve for receiving antiviral drugs during the 2009 influenza pandemic given by equation (2) (in Methods section) fit to National Household Travel Survey (NHTS) data on privately operated vehicle travel for the entire US underinsured population.

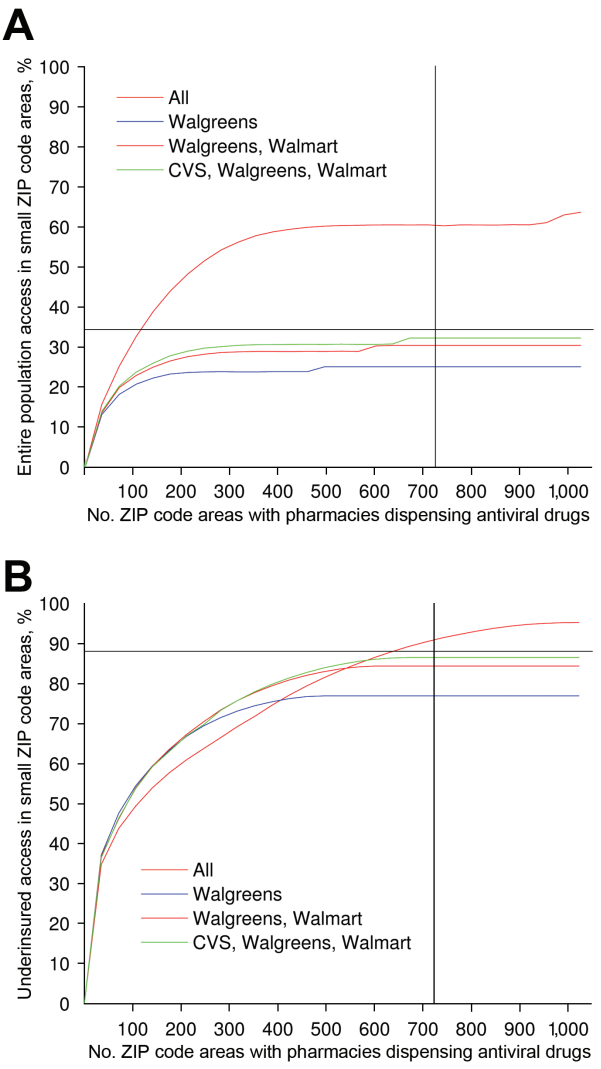

Figure 2. Antiviral drug access in underinsured populations achieved by the Texas antiviral drug distribution network during the 2009 influenza A pandemic and by optimized antiviral drug distribution networks, for A) small ZIP code (US postal code) areas (i.e., ZIP code areas with <1,000 underinsured persons) and B) statewide. Access is the expected fraction of the underinsured population willing to travel to the nearest dispensing pharmacy to obtain antiviral drugs. The black vertical and horizontal lines indicate the number of ZIP code areas that participated in the Texas 2009 distribution network and the estimated access achieved, respectively. For each network size (number of dispensing ZIP code areas), a hybrid optimization was performed to maximize coverage in small ZIP code areas and overall (see Methods for details). Color indicates which combination of 13 major pharmacy chains plus independents were considered in the optimization. For a distribution network of size 723 (comparable to the Texas 2009 H1N1 antiviral drug distribution), the best performing single-chain (Walgreens), 2-chain combination (Walgreens and Walmart), and 3-chain combination (Walgreens, Walmart, and CVS) provided near optimal coverage statewide, but critically underserved the smallest ZIP code areas.

Table 2. Expected access for antiviral drugs during the 2009 influenza pandemic provided by 3 drug distribution networks, Texas, USA*

Characteristic	Texas 2009 network	Optimized network†	All pharmacies network
Small ZIP code area access, %	34.5	60.5	63.8
Statewide access, %	88.0	90.8	95.2
No. ZIP code area dispensing points	723	723	1,023
Population living within dispensing ZIP code areas, %	76.5	79.3	91.8
Average miles traveled outside ZIP code area (SD)‡	4.5 (3.8)	3.8 (3.1)	5.7 (4.0)
Median miles traveled outside ZIP code area§	3.0	2.6	4.2

*ZIP code area, US postal code area. A small ZIP code area is an area with <1,000 underinsured persons.
†Initially, we optimized 75% of dispensing points (542) to maximize access solely in small ZIP code areas and recorded the access achieved. We then optimized all 723 dispensing points to maximize statewide access and constrained the solution to achieve ≥95% of the small ZIP code area access achieved in the initial optimization.
‡Population-weighted average travel distance to nearest dispensing pharmacy considering only ZIP code areas without their own dispensing pharmacies. When all pharmacies dispense antiviral drugs (fourth column), the longer average distance is an artifact of few persons living in ZIP code areas without pharmacies.
§Population-weighted median travel distance to nearest dispensing pharmacy, considering only ZIP code areas without their own dispensing pharmacies. When all pharmacies dispense antiviral drugs (fourth column), the longer median distance is an artifact of few persons living in ZIP code areas without pharmacies.

to each pharmacy, or the capacity of individual pharmacies. Additional details on methods are available at http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf.

The Web-based decision-support tool based on this model provides solutions for a range of values of *b* (Figure 2) and displays the trade-off between the expected access for the target population and the number of dispensing points. This tool also enables the user to select specific solutions for further analysis.

We considered 3 types of objective functions, all of which focus exclusively on the underinsured population in Texas: maximizing statewide access, maximizing access in small ZIP code areas (i.e., ZIP code areas with <1,000 underinsured persons), and a hybrid that combines the first 2 objectives. For our hybrid optimization model, we first specified a percentage of all dispensing points to focus on small ZIP code areas (P). Second, we optimized P of all dispensing points solely for access in small ZIP code areas and recorded the access achieved in small ZIP code areas (A_s). Third, we started over and optimized all dispensing points by using the statewide objective function with the added constraint that the solution must achieve a minimum of $0.95A_s$ access in small ZIP code areas. This method simultaneously achieves near maximal coverage statewide and in small ZIP code areas.

Results

During the 2009 influenza pandemic, the Texas DSHS recruited 1,393 pharmacies from 6 major chains and 71 independent pharmacies to dispense antiviral drugs from the SNS and state cache to underinsured populations. These pharmacies were located in 723 of the 1,023 ZIP code areas in Texas that had ≥1 pharmacy. We estimated that

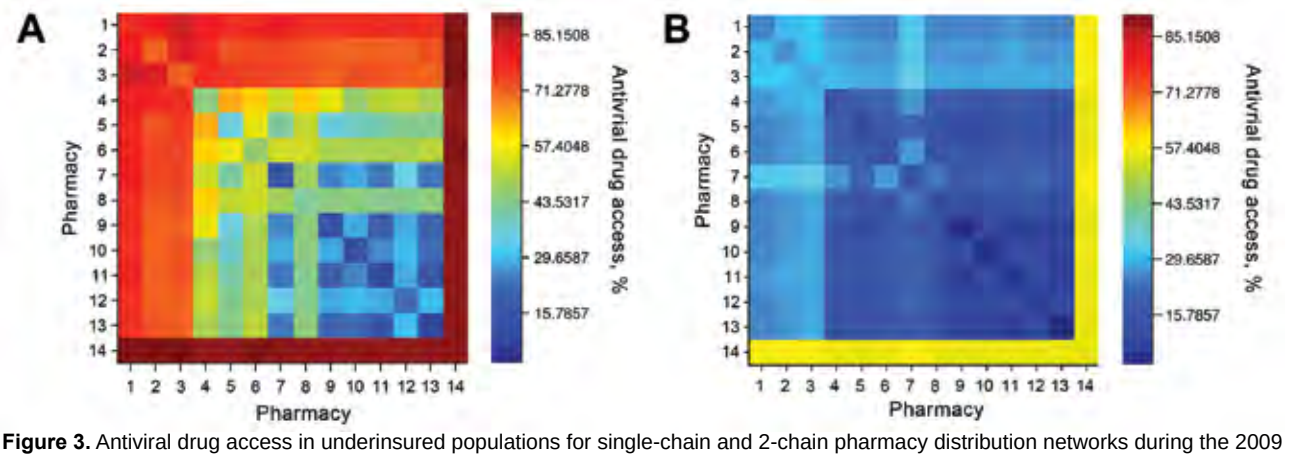

Figure 3. Antiviral drug access in underinsured populations for single-chain and 2-chain pharmacy distribution networks during the 2009 influenza pandemic, Texas, USA. Each network contains a maximum of 723 distribution points, and was designed by using a hybrid optimization that maximizes coverage in small ZIP code (US postal code) areas and overall (see text for details). Color indicates the expected percentage of the underinsured population willing to travel to dispensing pharmacies to obtain antiviral drugs A) statewide and B) in small ZIP code areas. Numbers along the baselines and the y-axes indicate single-chain networks. 1, Walgreens; 2, CVS; 3, Walmart; 4, HEB; 5, Kroger; 6, Target; 7, Brookshire; 8, Sam's Club; 9, Tom Thumb; 10, Randalls; 11, United; 12, Costco; 13, Kmart; 14, Independents (independent pharmacies).

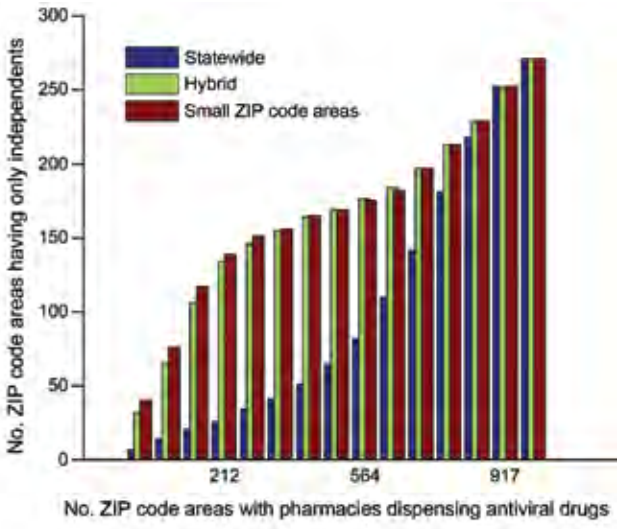

Figure 4. Number of sites in the antiviral drug distribution network during the 2009 influenza pandemic that contained only independent pharmacies (independents; i.e., no major chains) when optimizing for the underinsured population in small ZIP code (US postal code) areas (i.e., ZIP code areas with <1,000 underinsured persons), statewide, or both (hybrid), Texas, USA.

this network provided antiviral drug access for 88% of the state's underinsured population (Figure 2). In comparison, we also estimated that optimization over all possible pharmacy chains produced a network expected to achieve comparable access by using only 526 ZIP code areas, increased access to 92.5% with 723 ZIP code areas, and reached a maximum access of 95.2% with all 1,023 ZIP code areas.

However, optimizing for statewide access can lead to critical gaps in coverage. We categorized all Texas ZIP code areas on the basis of underinsured population sizes into small (<1,000 persons), medium (1,001–7,000 persons), and large (>7,000 persons). These areas contained 7%, 51%, and 42% of the statewide underinsured population, respectively. The actual Texas 2009 distribution network and the corresponding optimized network (with 723 ZIP code areas) were estimated to achieve only 34.5% and 38.3% access in small ZIP code areas, respectively, but reached 88.0% and 92.5% access overall. By definition, the small ZIP code areas do not carry much weight in a statewide optimization model. They also tend to be more remote than larger ZIP code areas, and thus have lower access to selected pharmacies.

To address this gap, we modified the objective function to maximize access specifically in small ZIP code areas. Although these modifications improved coverage in these hard-to-reach populations, the solutions were suboptimal overall. Thus, we developed a hybrid optimization procedure that sequentially ensures high access statewide and in small ZIP code areas. With 723 dispensing points, the hybrid method with $P = 75\%$ of dispensing points allocated to small ZIP code areas produced networks that are expected to achieve 60.5% access in small ZIP code areas and 90.5% overall. For comparison, the highest possible access (when all pharmacies in the state dispense antiviral drugs) was estimated to reach 63.8% and 95.2% in the 2 populations, respectively (Table 2). For populations living in ZIP code areas without pharmacies dispensing antiviral drugs, optimization reduced the average travel distance to the nearest dispensing pharmacy from 4.5 miles to 3.8 miles.

A state might opt to limit the number of chains in the distribution network to simplify logistics. The pharmacy chains eligible for participation in Texas include Brookshire, Costco, CVS, HEB, Kmart, Kroger, Randalls, Sam's Club, Target, Tom Thumb, United, Walgreens, Walmart, and independent retail pharmacies (henceforth independents).

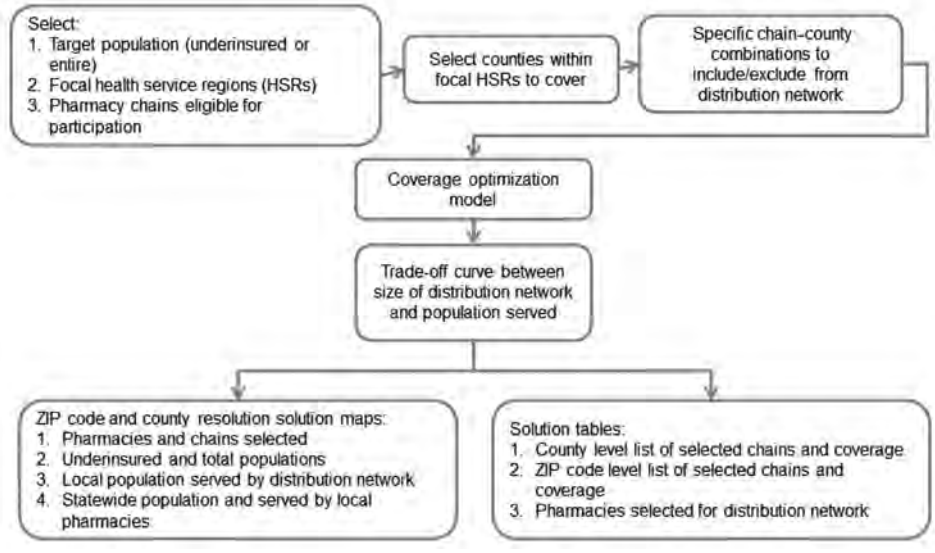

Figure 5. Flowchart of the antiviral drug distribution decision support tool during the 2009 influenza pandemic (*28*), Texas, USA.

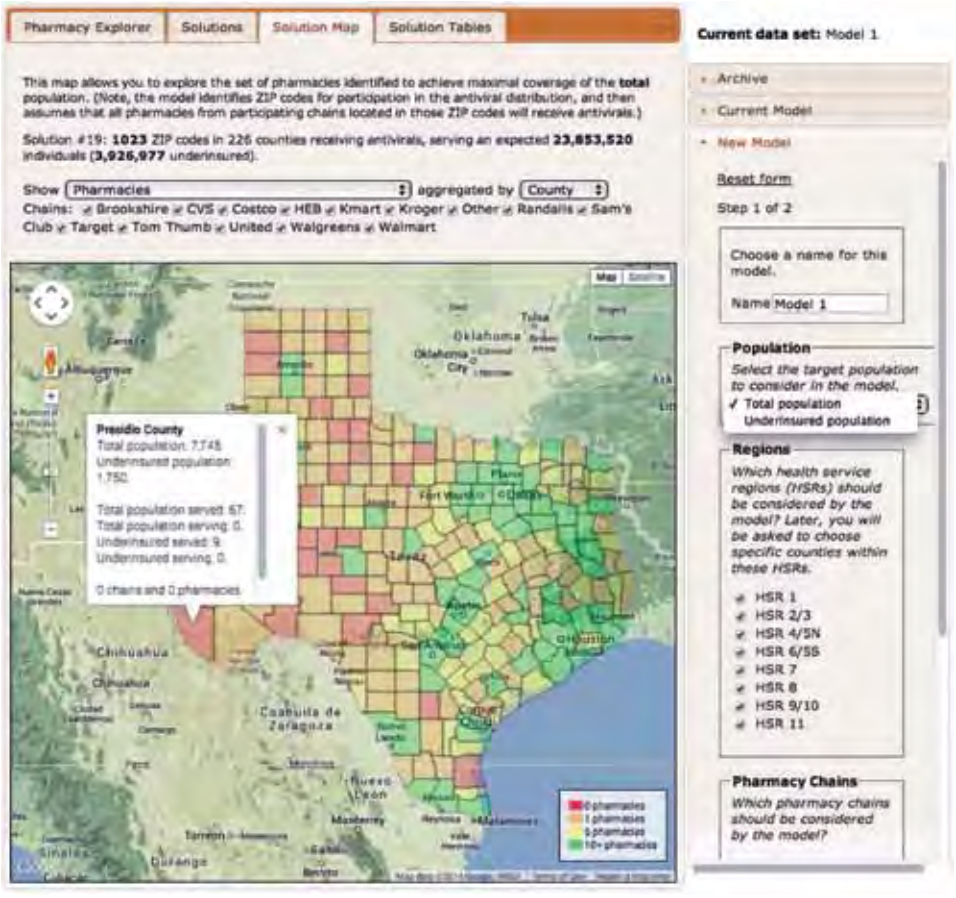

Figure 6. Screenshot of the antiviral drug distribution decision support tool used during the 2009 influenza pandemic (*28*), Texas, USA.

During the 2009 influenza pandemic, the Texas DSHS distributed antiviral drugs from the SNS and state cache through 6 major chains (Brookshire, HEB, Kroger, United, Walgreens, and Walmart), and independents. When we restricted the optimization to a few major chains, the resulting networks still achieved broad statewide coverage (Figures 2, 3). For example, Walgreens alone was expected to achieve ≈75% coverage if it dispenses in all of its 490 ZIP code areas; CVS and Walmart followed close behind (located in 422 and 372 ZIP code areas, respectively). These 3 chains have the greatest presence in the state (Table 1), but have highly overlapping geographic areas. Broad accessibility can also be achieved through a combination of smaller chains with geographic complementarity, for example, HEB and Kroger (in 189 and 167 ZIP code areas, respectively). Walgreens and Walmart overlap over half of their ZIP code areas (256), whereas HEB and Kroger overlap in only 30 ZIP code areas. However, the number of ZIP code areas alone is not predictive of access. For example, Brookshire has almost as many stores as Target (in 118 and 127 ZIP code areas, respectively), yet provides considerably less statewide access alone and in combination with other stores.

However, the major chains did not reach the underinsured populations in the small ZIP code areas, even under the hybrid optimization that explicitly targets these hard-to-reach populations (Figure 2, panel A). Independent pharmacies are essential to bridging this gap in coverage. The maximum access achieved by a 2-chain combination in small ZIP code areas is only 33% (Brookshire and Walmart) (Figure 3). Under the hybrid objective, optimized networks with <500 dispensing points yield solutions for all major pharmacy chains plus independents that provided slightly lower statewide accessibility than the corresponding solutions for major chains (Figure 2, panel B), in exchange for higher coverage in small ZIP code areas (Figure 2, panel A). Of the 1,023 ZIP code areas with ≥1 pharmacy, 271 have only independent pharmacies. Of these pharmacies, 167 are in small ZIP code areas, and were typically selected when optimizing for access in small ZIP code areas, but not when optimizing for access statewide (Figure 4).

Discussion

Many states plan to enlist commercial pharmacies in the dispensing of SNS antiviral drugs during future influenza pandemics (*20–22*,*34*). We have developed and

demonstrated a simple, extensible, facility-location model for designing pharmacy-based antiviral drug distribution networks that effectively reach target populations. This model has been parameterized for the state of Texas and incorporated into a Web-based decision-support tool (*28*) for the Texas DSHS (Figures 5, 6). The user can opt to target the underinsured or total population statewide or within any specified counties and to exclude or include particular pharmacies and pharmacy chains. On the basis of user input, the tool solves a family of optimization models, spanning the full range of possible network sizes, and presents the structure and performance of the optimized networks by using interactive graphs and maps. The tool is designed for use by Texas DSHS staff, who have Web-based access to it. Although this implementation is specific for Texas, the general model structure is readily adaptable to other jurisdictions. Adaptation requires specification of geographic units (e.g., ZIP code areas or counties), distances between each pair of units, estimated target population sizes within each unit, and number of pharmacies from each eligible chain located within the unit. The model can be easily extended to other states by using data available through the US Census Bureau and state pharmacy associations (*29*,*35*).

The optimization model is driven by a willingness-to-travel model, which was estimated from NHTS data. The nature and resolution of available data led to several simplifying assumptions. After fitting several decaying functions to the data, we chose a simple model that considers only the distance between one's home ZIP code area and the nearest pharmacy ZIP code area, rather than, for example, a more complex gravity model that incorporates the attractiveness of a store (*36*,*37*). The national data did not specify health-related travel patterns. Texas NHTS data had coarser mileage bins, and represented only 1% of the nation-wide person-trips. Thus, we used national-scale data on all available travel categories (to earn a living, family/personal business, school/church, social/recreational, and miscellaneous), and included only travel by privately operated vehicles (because of the sparsity of data on public transportation and bicycles). We also approximated home ZIP code area distances to pharmacy ZIP code area distances by using great circle distances between the ZIP code centroids, rather than road travel distances between their street addresses, which can underestimate the distance an individual must travel. Finally, we approximated the underinsured and small ZIP code area populations by using methods described in the Data section.

During future pandemics, timely and effective deployment of antiviral drugs from the SNS and state cache might be essential for reducing early illness and death (*19*). Data-driven models, such as those in this optimization tool can be instrumental in the planning process, enabling public health agencies to identify and recruit networks of broad-reaching chains and remote independent pharmacies that can achieve equitable and effective distributions to target groups, such as underinsured, high-risk, or age-specific populations. This tool will also facilitate rapid, adaptive decision-making during pandemics, if, for example, a region requires additional supplies, particular pharmacies are unwilling or unable to provide assistance, or the target population changes.

As with many optimization studies, the general insights gleaned from the design and preliminary applications of this decision-support tool might be as valuable as the tool itself. The Texas DSHS has gained actionable perspectives on the geographic coverage and redundancies of major pharmacy chains, the unique reach of independent pharmacies, and as discussed further (http://www.bio.utexas.edu/research/meyers/_docs/publications/SinghEID14Supplement.pdf), the convenient overlap between optimal distribution networks for the underinsured population and total population. Given the need for and difficulties associated with enlisting independent pharmacies in sparsely populated areas, state agencies should engage them well before the next pandemic, perhaps in partnership with local health departments.

Acknowledgments

We thank Michael Poole for sharing data and providing first-hand perspectives on deployment of SNS antiviral drugs in Texas during the influenza pandemic during 2009.

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pH Level as a Marker for Predicting Death among Patients with *Vibrio vulnificus* Infection, South Korea, 2000–2011

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Release date: January 16, 2015; Expiration date: January 16, 2016

Learning Objectives

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

- Describe characteristics of patients infected with *Vibrio vulnificus*
- Assess the outcome of infection with *V. vulnificus*
- Distinguish significant prognostic factors among patients infected with *V. vulnificus*
- Identify the cutoff value for arterial pH with the best discretion for the prognosis of *V. vulnificus* infection

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Disclosures: ***Na Ra Yun, MD; Dong-Min Kim, MD, PhD; Jun Lee, MD;*** *and* ***Mi Ah Han, MD, PhD,*** *have disclosed no relevant financial relationships.*

Vibrio vulnificus infection can progress to necrotizing fasciitis and death. To improve the likelihood of patient survival, an early prognosis of patient outcome is clinically important for emergency/trauma department doctors. To identify an accurate and simple predictor for death among *V. vulnificus*–infected persons, we reviewed clinical data for 34 patients at a hospital in South Korea during 2000–2011; of the patients, 16 (47%) died and 18 (53%) survived. For nonsurvivors, median time from hospital admission to death was 15 h (range 4–70). For predicting death, the areas under the receiver operating characteristic curves of the Acute Physiology and Chronic Health Evaluation (APACHE) II score and initial pH were 0.746 and 0.972, respectively (p = 0.005). An optimal cutoff pH of <7.35 had a sensitivity of 100% and specificity of 83%. Compared with the APACHE II score, the initial arterial blood pH level in *V. vulnificus–infected* patients was a more accurate predictive marker for death.

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Vibrio vulnificus is a motile, halophilic, rod-shaped gram-negative pathogen that lives in estuarine environments (*1*). During the last decade, the prevalence of *V. vulnificus* infection has increased worldwide (*2*–*4*). In the United States, the annual number of *V. vulnificus* infections reported to the 10-state Foodborne Disease Active Surveillance Network (http://www.cdc.gov/foodnet/) increased from 0.01 cases/100,000 persons in 1996 to 0.05 cases/100,000 persons in 2010 (*5*).

V. vulnificus infections, which are mainly characterized by skin and soft-tissue infections or septicemia, can develop a fulminant course (*1*,*6*). The severe form of *V. vulnificus* soft-tissue infection, necrotizing fasciitis, often has adverse outcomes, including death. In such cases, death can occur within 48 h of hospital admission, especially if the infection is associated with the development of sepsis or septic shock, which increases the case-fatality rate to 26%–71% (*7*–*11*). For cases of *V. vulnificus* infection caused by the consumption of contaminated shellfish, the death rate is ≈53%, and the rate is higher (67%) among patients with liver disease (*12*). The death rate may increase to 100% in patients with septicemia if treatment is delayed for 72 h after symptom onset (*13*).

Surgery, including fasciotomy, debridement, and limb amputation, early in *V. vulnificus* infection has been advocated by some as a means for improving patient outcomes (*3*,*10*,*14*). However, the proper timing for surgery is debatable: in the early stages of infection, patients may not be stable enough to be moved to the operating room, and patients may have little chance of surviving if surgery is delayed until a later stage of infection (*3*). Because *V. vulnificus* infections can be life threatening, it is critical for emergency/trauma department doctors, including surgeons who make decisions regarding surgery for necrotizing fasciitis, to have early knowledge of a patient's prognosis.

Some clinical data are available regarding the prognostic factors for patients with fatal *V. vulnificus* infection (*10*,*15*–*17*). Higher Acute Physiology and Chronic Health Evaluation (APACHE) II and Mortality in Emergency Department Sepsis (MEDS) scores have been reported to be significant prognostic indicators for *V. vulnificus*–infected patients (*10*,*15*). However, the APACHE II and MEDS scoring systems might be too complex to use quickly in an emergency setting. Thus, to find a marker to predict the risk for death among *V. vulnificus*–infected patients, we investigated the initial clinical and laboratory data available in hospital emergency departments. The aims of our study were to describe the clinical outcomes of *V. vulnificus*–infected patients and identify an accurate and simple predictive marker for death.

Materials and Methods

Patients

For the study, we identified 34 *V. vulnificus*–infected patients (≥18 years of age) who had been admitted during January 2000–December 2011 to Chosun University Hospital, a tertiary teaching hospital in Gwangju, South Korea. *V. vulnificus* infection was diagnosed on the basis of blood and/or wound culture results. Clinical isolates were identified by using a Vitek II automated system (bioMérieux, Marcy l'Étoile, France). The study was approved by the Ethics in Human Research Committee of Chosun University Hospital.

Data Collection and Definitions

Demographic information and data regarding patients' clinical manifestations, laboratory variables, operations,

Table 1. Demographic and clinical features for 34 patients in a study looking for predictors of death among persons with *Vibrio vulnificus* infection, South Korea, 2000–2011*

Variable	Nonsurvivors, n = 16†	Survivors, n = 18†	p value
Sex			0.346
M	15 (93.8)	15 (83.3)	
F	1 (6.3)	3 (16.7)	
Age, y	58 (43–74)	57 (45–67)	0.740
Interval, d, between symptom onset and hospital admission	3 (1–5)	3 (0–7)	0.304
Route of exposure			
Consumption of seafood	11 (69)	14 (78)	0.565
Wound exposure to seawater	3 (19)	2 (11)	0.723
Unknown	2 (12)	2 (22)	0.632
Underlying condition			
Chronic liver disease	15 (94)	17 (94)	0.872
Chronic alcoholism	13 (81)	14 (78)	0.803
Hepatitis B virus infection	2 (13)	3 (17)	0.732
Hepatitis C virus infection	0	0	NA
Signs and symptoms at hospital admission			
Any gastrointestinal symptom	7 (54.8)	5 (27.8)	0.331
Any skin and soft tissue lesion	12 (75.0)	15 (83.3)	0.549
Bacteremia present	10 (63)	8 (44)	0.292
Surgery performed	7 (44)	8 (44)	0.967
Appropriate antimicrobial drug treatment received	13 (81)	14 (78)	0.810

*NA, not applicable.
†Except for age, data are numbers (%) or medians (range).

Table 2. Laboratory results 34 patients in a study looking for predictors of death among persons with *Vibrio vulnificus* infection, South Korea, 2000–2011*

Variable	Reference values	Values for patients		p value
		Nonsurvivors, n = 16	Survivors, n = 18	
Hematologic values				
Leukocyte count/μL	4,000–8,000	6,721 (1,030–25,950)	9,687 (1,940–21,770)	0.167
Hemoglobin, g/dL	12.0–16.0	12.5 (7.3–17.8)	11.5 (8.2–15.5)	0.248
Platelets, 10^3/μL	150–400	62 (13–147)	81 (13–243)	0.330
PT, s	9.4–12.5	17.7 (10.5–30.4)	14.9 (10.2–19.5)	0.111
aPTT, s	28.0–44.0	48.5 (28.8–103.0)	37.4 (21.5–57.5)	0.040
Arterial blood gas analysis values				
pH	7.350–7.450	7.08 (6.82–7.36)	7.41 (7.22–7.50)	<0.001
HCO_3, mmol/L	21.0–28.0	8.4 (3.6–13.9)	18.6 (8.7–25.6)	<0.001
PO_2, mm Hg	83.0–108.0	103.4 (69.2–157.0)	86.6 (52.3–162.8)	0.035
PCO_2, mm Hg	35.0–45.0	26.7 (14.3–41.0)	28.8 (21.6–38.0)	0.314
Clinical chemistry values				
Albumin, g/dL	3.50–5.20	2.63 (2.07–3.00)	2.82 (1.66–3.84)	0.201
BUN, mg/dL	8.0–20.0	31.8 (10.0–56.9)	22.6 (9.6–49.7)	0.043
Creatinine, mg/dL	0.5–1.3	3.59 (1.52–5.90)	1.76 (0.60–3.90)	<0.001
Glucose, mg/dL	60–109	134 (22–306)	141 (40–300)	0.764
Bilirubin, mg/dL	0.2–1.2	7.5 (0.5–48.3)	2.3 (0.5–4.1)	0.094
Liver enzyme values				
AST, IU/L	5–40	443 (43–1,696)	111 (30–323)	0.006
ALT, IU/L	5–40	100 (22–290)	57 (17–133)	0.020
APACHE II score		18 (10–31)	14 (10–32)	0.036

*Data are expressed as median (range). ALT, alanine aminotransferase; APACHE, Acute Physiology and Chronic Health Evaluation; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen; HCO_3, serum bicarbonate; PCO_2, carbon dioxide partial pressure; PO_2, partial pressure of oxygen; PT, prothrombin time.

antimicrobial drug regimens, underlying diseases, and time to death were obtained by chart review. In Chosun University Hospital, arterial blood gas analysis and blood cultures are routinely performed before starting empirical antimicrobial drug treatment in patients with signs or symptoms of infection.

The use of an appropriate antimicrobial drug regimen was noted if a patient had been administered ceftriaxone, cefotaxime, or ciprofloxacin alone or in combination with doxycycline. Chronic liver disease was noted if a patient had shown clinical or laboratory signs of chronic liver disease caused by alcoholism or by hepatitis B or C virus. Fasciotomy was noted if surgery had been performed for necrotizing fasciitis. Death was defined as dying while in the hospital.

Statistical Analyses

We compared demographic and clinical characteristics for patients who survived and those who did not by using *t*-tests for continuous variables and χ^2 tests for categorical variables. For APACHE II scores and pH levels at admission, we calculated the areas under the receiver operating characteristic curves (AUROCs) for predicting death caused by *V. vulnificus* infection. Sensitivities, specificities, positive predictive values, negative predictive values, positive likelihood ratios (how much to increase the probability of survival if the test is positive), and negative likelihood ratios (how much to decrease the probability of survival if the test is negative) of various APACHE II scores and pH cutoff levels on admission were calculated (*18*). The Youden index was used to determine the optimal cutoff value (*19*).

Multivariate analyses were performed by using the Cox proportional hazards model; variables that showed a p value of <0.05 in univariate analyses were included in these analyses. Of the variables for arterial blood gas analysis, pH was the only variable included in the model, and the APACHE II score was excluded from the multivariate model because of collinearity with pH and creatinine. Survival curves were constructed by using the Kaplan–Meier method, and a log-rank test was used for comparison. Survival was measured from the time of hospital admission to the time of death. We evaluated death status as death

Table 3. Prognostic factors for death, as determined by multivariate analysis, among patients with *Vibrio vulnificus* infection, South Korea, 2000–2011*

Variable	Relative risk (95% CI)	p value
pH (per 0.1 increase)	0.441 (0.305–0.637)	<0.001
Creatinine (per mg/dL increase)	2.114 (1.105–4.043)	0.023
BUN (per mg/dL increase)	1.046 (0.981–1.115)	0.171
AST (per IU/L increase)	0.998 (0.995–1.001)	0.221
ALT (per IU/L increase)	1.014 (0.991–1.039)	0.236
aPTT (/s increase)	0.983 (0.935–1.033)	0.496

*ALT, alanine aminotransferase; aPTT, activated partial thromboplastin time; AST, aspartate aminotransferase; BUN, blood urea nitrogen.

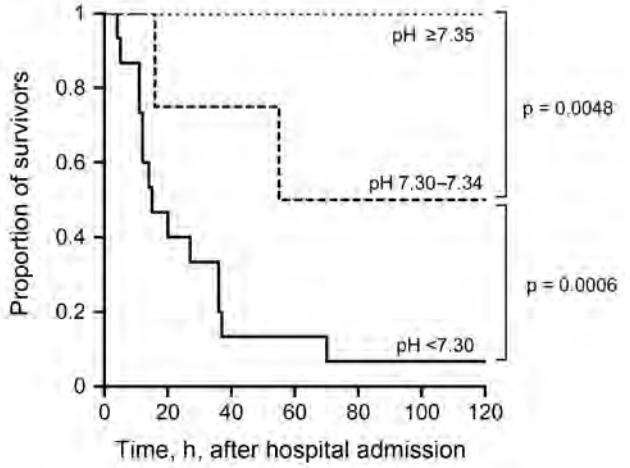

Figure 1. Survival curves of patients, by pH level at hospital admission, in a study investigating predictors of death among persons with *Vibrio vulnificus* infection, South Korea, 2000–2011.

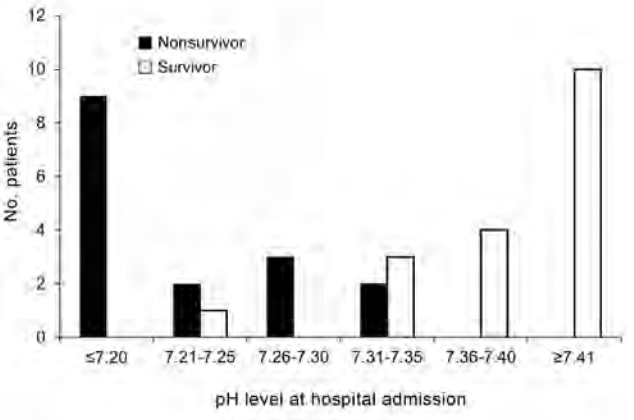

Figure 2. Numbers of surviving and nonsurviving patients, by pH level at hospital admission, in a study investigating predictors of death among persons with *Vibrio vulnificus* infection, South Korea, 2000–2011.

occurring ≤120 h after admission; patients who lived >120 h after admission were not included in the death statistics. A 2-tailed p value of <0.05 was considered to indicate statistical significance. All statistical analyses were performed by using SAS software, version 8.2 (SAS Institute Inc., Cary, NC, USA).

Results

Clinical Outcomes for Patients with *V. vulnificus* Infection

Of the 34 patients with a diagnosis of V. vulnificus infection, 3 (9%) were female and 31 (91%) were male. A total of 25 patients had a history of consuming raw seafood, 5 had a wound exposure to seawater, and 4 were unaware of their exposure source. Of the 32 patients who had chronic liver disease, 27 (84%) had chronic alcoholism and 5 (16%) had hepatitis B virus infection.

Of the 34 total patients, 16 (47%) died and 18 (53%) survived. The median time between arrival at the hospital and death was 15 h (range 4–70 h). For patients who survived and those who did not, the differences in underlying diseases, interval between symptom onset and hospital admission, initial appropriate antimicrobial drug treatment, and surgical treatment were not significant (Table 1).

Predictive Markers for Death

Univariate analysis showed that activated partial thromboplastin time and levels of pH, partial pressure of oxygen, serum bicarbonate, blood urea nitrogen, creatinine, aspartate aminotransferase, and alanine aminotransferase were significantly different between patients who survived and those who did not survive (Table 2). In multivariate analysis, initial pH on arterial blood gas analysis and serum creatinine level were significant risk factors for death (Table 3).

We found a significant difference in survival for patients with different arterial pH levels at hospital admission (Figure 1). No patients with a pH level of <7.2 at admission survived; all patients with a pH level of ≥7.35 survived (Figure 1, 2). An optimal pH cutoff level of <7.35 had a sensitivity of 100%, specificity of 83%, positive predictive value of 84%, negative predictive value of 100%, positive likelihood ratio of 6.0, and negative likelihood ratio of 0. An optimal APACHE II cutoff score of ≥14 had a sensitivity of 75%, specificity of 67%, positive predictive value of 67%, negative predictive value of 75%, positive likelihood ratio of 2.3, and negative likelihood ratio of 0.3 (Table 4).

Table 4. Predictors of death among patients with *Vibrio vulnificus* infection, South Korea, 2000–2011*

Predictor	Sensitivity, %	Specificity, %	Predictive value, %		Likelihood ratio	
			Positive	Negative	Positive	Negative
pH level						
<7.30	87.5	94.4	93.3	89.5	15.63	0.13
<7.35	100.0	83.3	84.2	100.0	5.99	0
<7.40	100.0	61.1	69.6	100.0	2.57	0
APACHE II score						
≥13	87.5	61.1	66.7	84.6	2.25	0.20
≥14	75.0	66.7	66.7	75.0	2.25	0.37
≥15	75.0	77.8	75.0	77.8	3.38	0.32

*APACHE, Acute Physiology and Chronic Health Evaluation.

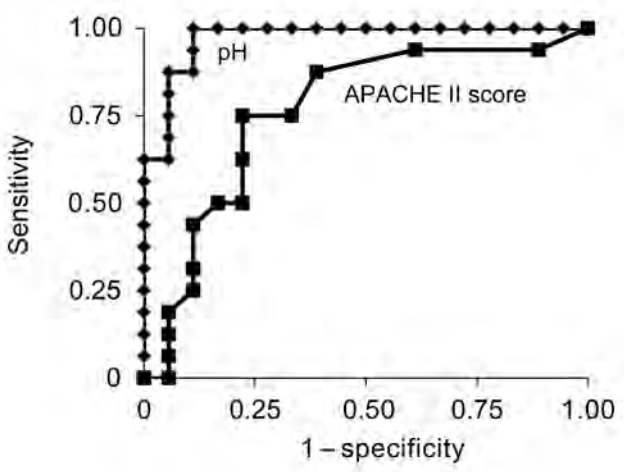

Figure 3. Receiver-operating characteristic curves (AUROCs) for pH level and Acute Physiology and Chronic Health Evaluation (APACHE) II score in a study investigating predictors of death among patients with *Vibrio vulnificus* infection, South Korea, 2000–2011. AUROC (95% CIs): pH level, 0.972 (range 0.924–1.000); APACHE II score,0.746 (range 0.595–0.933) (p = 0.005).

The AUROC of pH for prediction of death was 0.972 (95% CI 0.924–1.000), and the AUROC of the APACHE II score was 0.746 (95% CI 0.595–0.933) (p = 0.005) (Figure 3).

Discussion

In this study, approximately half of the patients with *V. vulnificus* infection died, and death occurred ≤72 h after hospital admission. Patient survival differed significantly by pH level at hospital admission, and the initial pH level for patients was a more accurate predictive marker for death than was the APACHE II score.

Many studies have asserted that antimicrobial drugs should be immediately administered to patients with suspected *V. vulnificus* infection (*3*,*8*,*15*,*20*). Other studies suggest that a combination of antimicrobial drugs (i.e., third-generation cephalosporin, ciprofloxacin, and doxycycline) is the best treatment for *V. vulnificus* infection (*21*,*22*). In addition, surgical intervention is necessary to remove necrotic tissue and bacteria (*20*). Survival of patients with necrotizing fasciitis has been shown to improve when adequate debridement and fasciotomy are performed early in infection (*3*,*20*,*23*,*24*).

However, in our study there was no difference in the time from symptom onset to hospital admission and the timing of the initiation of appropriate antimicrobial drug treatment and surgical treatment for patients who survived and those who did not. This lack of difference may partly be due to the fact that patients died too early after admission to benefit from antimicrobial drug treatment or surgery. The median time to death for nonsurviving patients in our study was 15 h, even though they arrived at the hospital relatively early after symptom onset. The time to death in our study seems to be shorter than that in another study, which showed 11 (73%) of the 15 deaths occurred <72 h after arrival at the hospital (*15*).

That earlier study and another study reported that APACHE II and MEDS scores at hospital admission were useful prognostic indicators in primary septicemia or wound infections caused by *V. vulnificus* (*10*,*15*). In another study, the level of *V. vulnificus* DNA was substantially higher in nonsurvivors than in survivors of *V. vulnificus* septicemia, and the *V. vulnificus* DNA load correlated with the APACHE II score (*17*). Our data also showed that the APACHE II score may be useful for prediction of death in *V.* vulnificus–infected patients, although the AUROC for the APACHE II score was significantly less than that for initial pH.

In previous reports, hemorrhagic bullae formation, necrotizing fasciitis, and septic shock were risk factors for death in patients with *V. vulnificus* infection (*8*,*25*–*27*). Although in our study, no skin or soft tissue lesions were associated with death, the pH and creatinine levels at hospital admission were independently associated with death of *V. vulnificus*–infected patients. Low arterial pH level might result from lactic acidosis caused by marked tissue hypoperfusion in shock and damage to extremities by necrotizing fasciitis (*28*,*29*). The finding that high creatinine level was associated with death also supports the possibility that hypoperfusion or sepsis contributed to the death of patients in this study because azotemia might result from renal hypoperfusion or septic renal injury.

Our study did have some limitations because of its retrospective design and the relatively small number of study patients, which may hamper generalization of the results. In addition, because most patients in this study had chronic liver disease, the study findings might not be applicable to patients with various concurrent diseases other than chronic liver disease.

Despite these limitations, our study may have relevant clinical implications. Our data demonstrate that the pH level determined by arterial blood gas analysis at hospital admission is more accurate than the APACHE II score as a marker for predicting death in *V. vulnificus*–infected patients. Arterial blood gas levels are easy to determine, and results are quickly available; thus, this test is useful in emergency or trauma departments.

We propose that arterial pH level should be determined for patients with suspected *V. vulnificus* infection as soon as possible after hospital admission. When the arterial pH level is low (<7.2), patient outcome may be unsatisfactory, regardless of emergency surgery and appropriate antimicrobial drug therapy. When the pH level is not low, patient outcome may be excellent if the patient receives appropriate antimicrobial drug treatment and, if needed, surgery.

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Refining Historical Limits Method to Improve Disease Cluster Detection, New York City, New York, USA

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Since the early 2000s, the Bureau of Communicable Disease of the New York City Department of Health and Mental Hygiene has analyzed reportable infectious disease data weekly by using the historical limits method to detect unusual clusters that could represent outbreaks. This method typically produced too many signals for each to be investigated with available resources while possibly failing to signal during true disease outbreaks. We made method refinements that improved the consistency of case inclusion criteria and accounted for data lags and trends and aberrations in historical data. During a 12-week period in 2013, we prospectively assessed these refinements using actual surveillance data. The refined method yielded 74 signals, a 45% decrease from what the original method would have produced. Fewer and less biased signals included a true citywide increase in legionellosis and a localized campylobacteriosis cluster subsequently linked to live-poultry markets. Future evaluations using simulated data could complement this descriptive assessment.

Detecting aberrant clusters of reportable infectious disease quickly and accurately enough for meaningful action is a central goal of public health institutions (*1*–*3*). Clinicians' reports of suspected clusters of illness remain critical for surveillance (*4*), but the application of automated statistical techniques to detect possible outbreaks that might otherwise not be recognized has become more common (*5*). These techniques are particularly important in jurisdictions that serve large populations and receive a high volume of reports because manual review and investigation of all reports are not feasible.

Challenges such as lags in reporting and case classification and discontinuities in surveillance case definitions, reporting practices, and diagnostic methods are common across jurisdictions. These factors can impede the timely detection of disease clusters. Statistically and computationally simple methods, including historical limits (*6*), a log-linear regression model (*7*), and cumulative sums (*8*), each have strengths and weaknesses for prospective cluster detection, but none adequately address these common data challenges. As technology advances, statistically and computationally intensive methods have been developed (*2*,*3*,*5*,*9*–*12*), and although these methods might successfully correct for biases, many lack the ease of implementation and interpretation desired by health departments.

Since 1989, the US Centers for Disease Control and Prevention has applied the historical limits method (HLM) to disease counts and displayed the results in Figure 1 of the Notifiable Diseases and Mortality Tables in the Morbidity and Mortality Weekly Report (*13*). Because the method relies on a straightforward comparison of the number of reported cases in the current 4-week period with comparable historical data from the preceding 5 years, its major strengths include simplicity, interpretability, and implicit accounting for seasonal disease patterns. These strengths make it a potentially very useful aberration-detection method for health departments (*12*,*14*–*18*). The Bureau of Communicable Disease (BCD) of the New York City (NYC) Department of Health and Mental Hygiene (DOHMH) implemented the HLM in the early 2000s ($HLM_{original}$) as a weekly analysis for all reportable diseases for which at least 5 years of historical data were available.

In $HLM_{original}$, 4 major causes of bias existed: 1) inconsistent case inclusion criteria between current and historical data; 2) lack of adjustment in historical data for gradual trends; 3) lack of adjustment in historical data for disease clusters or aberrations; and 4) no consideration of reporting delays and lags in data accrual. Our objectives were to develop refinements to the HLM ($HLM_{refined}$) that preserved the simplicity of the method's output and improved its validity and to characterize the performance of the refined method using actual reportable disease surveillance data. Although we describe the specific process for refining BCD's aberration-detection method, the issues presented are common across jurisdictions, and the principles and results are likely to be generalizable.

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Methods

Overview of Disease Monitoring at BCD

BCD monitors ≈70 communicable diseases among NYCs 8.3 million residents (*19*). For passive surveillance, laboratories and providers are required to submit disease reports (*20*), and these reports flow into a database system (Maven, Consilience Software, Austin, TX, USA). Each case is classified into 1 of 12 case statuses (Table 1). Depending on the disease, cases initially might be assigned a transient pending status and, upon investigation, be reclassified as a case (confirmed, probable, or suspected) or "not a case." For each disease, a designated disease reviewer is responsible for reviewing cases.

HLM Overview

HLM compares the number of reported cases diagnosed in the past 4 weeks (X_0) with the number diagnosed within 15 prior periods ($X_{1–15}$) comprising the same 4-week period, the preceding 4-week period, and the subsequent 4-week period during the past 5 years (Figure 1). A 4-week temporal unit of analysis balances timeliness with stability (*6*,*21*). For any given disease, if the ratio of current counts to the mean of the fifteen 4-week totals is greater than historical limits, then the current period is considered aberrant (i.e., a signal is generated) (online Technical Appendix (http://wwwnc.cdc.gov/EID/article/21/2/14-0098-Techapp1.pdf). In applying this method in NYC, only increases in case counts >2 SD above the historical mean are considered because artifactual decreases in case counts would be detected by separate quality-control measures.

$HLM_{original}$ was run each Monday for the 4-week interval that included cases diagnosed through the most recent Saturday. Data on confirmed, probable, suspected, or pending cases (Table 1) were analyzed at 3 geographic resolutions: citywide, borough (5 boroughs), and United Hospital Fund (UHF) neighborhood (42 neighborhoods). UHF neighborhoods are aggregations of contiguous ZIP codes used to define communities (*22*). Data were analyzed at the 2 subcity geographic resolutions to improve the signal-to-noise ratio for spatial clusters. For a signal to be generated, the current period was required to contain at least 3 cases, and the ratio of cases to the historical mean was required to be greater than historical limits. Disease reviewers were promptly notified of any signals and were provided with a corresponding case line list.

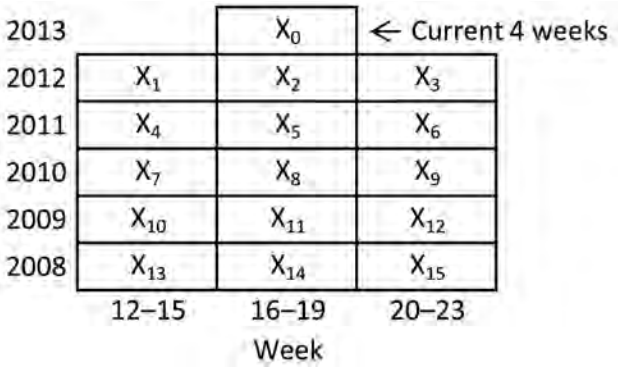

Figure 1. Following Stroup et al. (*21*), a schematic of the periods included in analyses using the historical limits method.

Table 1. Case statuses in current and baseline periods included in $HLM_{original}$ and $HLM_{refined}$, New York City, New York, USA*

Case status	Included in $HLM_{original}$	Included in $HLM_{refined}$
Confirmed	Yes	Yes
Probable	Yes	Yes
Suspected	Yes	Yes
Pending†	Yes	Yes
Unresolved	No	Yes
"Not a case"	No	Yes
Chronic carrier	No	Yes
Asymptomatic infection	No	Yes
Seroconversion 1 y	No	Yes
Not applicable	No	Yes
Contact	No	No
Possible exposure	No	No

*HLM, historical limits method; $HLM_{original}$, method as originally applied in New York City before May 20, 2013; $HLM_{refined}$, refined method applied starting May 20, 2013.
†Pending is a transient status that in the normal course of case investigations can be assigned to a case in the current period but not in the baseline period.

Refinements to Address Biases

Bias 1: Inconsistent Case Inclusion Criteria

The first limitation of $HLM_{original}$ as applied in NYC was that case inclusion criteria caused current disease counts to be systematically higher than baseline disease counts for many diseases. Cases classified as confirmed, probable, suspected, or pending were analyzed, but some cases with an initial pending status were ultimately reclassified after investigation as "not a case." This reclassification process was complete for historical periods but ongoing for the current period.

The proportion of initially pending cases that were reclassified to confirmed, probable, or suspected (rather than "not a case") varied widely by disease (Figure 2). For diseases for which this confirmatory proportion was low, the disease counts in the current period included a high proportion of pending cases that would ultimately be reclassified as "not a case," leading to false signals (type I errors). A similar bias might apply for nationally notifiable data in that provisional and final case counts may be systematically different (*23*).

Refinement 1: Consistent Case Inclusion Criteria

$HLM_{refined}$ included almost all reported cases in the analysis regardless of current status (Table 1). This simple modification led to a more valid comparison of total reporting

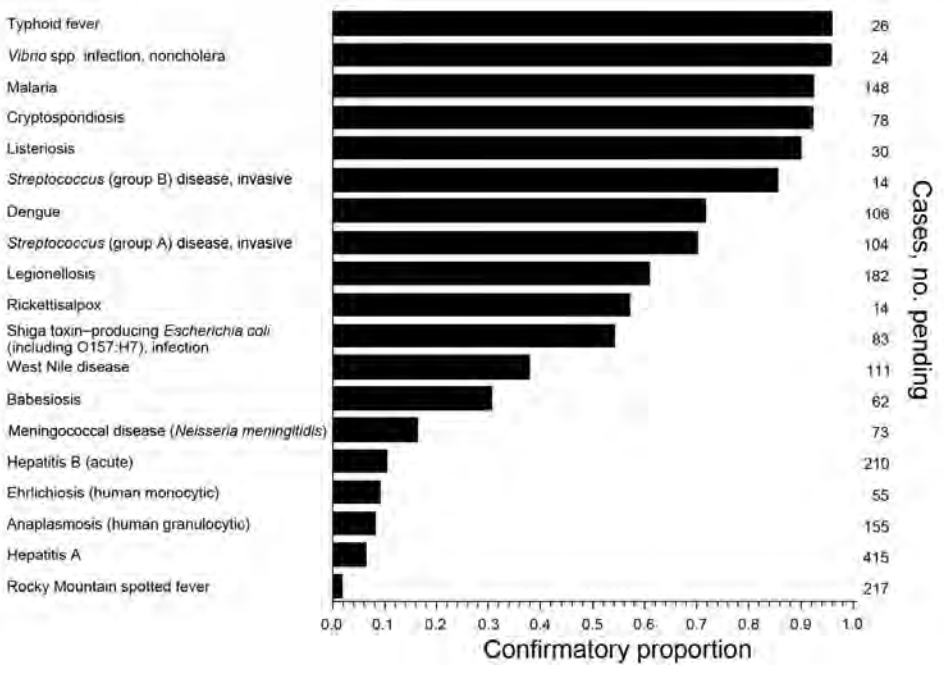

Figure 2. Confirmatory proportion of pending cases for diseases with any pending cases, New York City, New York, USA, July–December 2012. The confirmatory proportion was defined as the proportion of initially pending cases that were reclassified to confirmed, probable, or suspected (rather than to "not a case"). Diseases that are not routinely investigated, e.g., campylobacteriosis, enter the database with confirmed (not pending) case status and are not shown.

volume between current and historical periods, assuming that reporting is consistent over time, rather than biased estimates of the true level of disease. We maintained the requirement of the presence of at least 3 confirmed, probable, suspected, or pending cases to be considered a signal to prevent alerts driven by cases classified as "not a case."

Bias 2: Gradual Trends in Historical Data

The second limitation of $HLM_{original}$ was the existence of increasing or decreasing trends over time in historical data for many diseases. Whether these trends are true changes in disease incidence or artifacts of changing reporting or diagnostic practices, anything that causes disease counts in the baseline period to be systematically higher than current disease counts increases type II errors, and anything that causes baseline disease counts to be systematically lower than current disease counts increases type I errors.

Refinement 2: Adjusted Historical Data to Remove Gradual Trends

For $HLM_{refined}$, we identified and removed any significant linear trend in historical data. We accomplished this refinement by running a linear regression on weekly case counts for each disease at each geographic resolution and refitting the resulting residuals to a trend line with a slope of 0 and an intercept set to the most recent fitted value. Across diseases, linear trends were of relatively small magnitude; the greatest was for *Campylobacter*, for which the slope increased by ≈0.25 cases per week (Figure 3).

To minimize the influence of outliers on the overall trend, we excluded weekly counts >4 SD above or below the average for the baseline period from the regression. However, these counts were added back after the model had been fitted.

Bias 3: Inclusion of Past Clusters in Historical Data

The third major bias in $HLM_{original}$ was the inclusion of past clusters or aberrations in historical data. This bias reduced the method's ability to detect aberrations going forward, which increased type II errors.

Refinement 3: Exclusion of Past Clusters from Historical Data

To prevent this bias, after adjusting for gradual trends, we considered any 4-week period in which disease counts were >4 SD above the average to be an outlier and reset the count to the average number of cases in the remaining historical instances of that 4-week period. (We selected the threshold of 4 SD after manually reviewing case counts over time for all diseases.) For example, during 2007–2011, the number of dengue fever cases diagnosed during weeks 35–38 in 2010 was >4 SD above the average number of cases during those 5 years. Consequently, that 4-week period in 2010 was considered an outlier and reset to the average dengue fever count in weeks 35–38 in 2007, 2008, 2009, and 2011 (Figure 4). This technique can cause the case counts over time to appear jagged, but because our objective was to ensure a valid comparison between historical and current data, the smoothness of trends over time is irrelevant.

Bias 4: Delays in Data Accrual

Finally, data accrual delays can contribute to type II errors. This method is applied on Mondays for the 4-week period

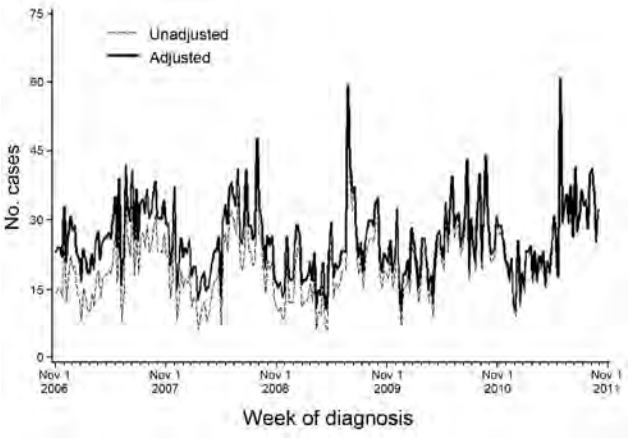

Figure 3. Unadjusted and adjusted weekly citywide counts of campylobacteriosis cases to illustrate adjustment for a linear trend in historical data, New York City, New York, USA, November 2006–October 2011.

that includes cases diagnosed through the most recent Saturday, so any lag between diagnosis and receipt by BCD of >2 days has the potential to deflate disease counts in the current period and reduce signal sensitivity. During July 18, 2012–August 28, 2013, the median lag between diagnosis and receipt by BCD was 5 days (range in median lag by disease 0–24 days).

Although DOHMH works with laboratories and providers to improve reporting practices, substantial reporting lags will continue for some diseases because of practices related to testing (e.g., time required for culturing and identifying *Salmonella* from a clinical sample) and surveillance (e.g., for some diseases, reports are held for delivery to the surveillance database until both a positive screening test and a confirmatory test are reported).

Refinement 4: Repeated Analyses to Accommodate Delays in Data Accrual

For diseases for which a delay of ≥1 week is not too long for a signal to be of public health value, we repeated the analysis for a given 4-week period over 4 consecutive weeks to allow for data accrual, thus improving signal sensitivity. In other words, we first analyzed cases diagnosed during a 4-week period on the following Monday. Updated data for the same 4-week period were re-analyzed on the subsequent 4 Mondays as data accrued to identify any signals that were initially missed because of incomplete case counts.

Customization by Disease

In $HLM_{original}$, we conducted the same analysis for all diseases under surveillance, despite very different disease agents and epidemiologic profiles. We solicited comments from disease reviewers to ensure that the method was being applied meaningfully to all diseases and received feedback that $HLM_{original}$ produced an unmanageable number of signals, which led to their dismissal without investigation. We also suspect that on some occasions $HLM_{original}$ did not detect true clusters because trends in disease counts decreased over the baseline period or because historical outbreaks masked new clusters. We responded by allowing for disease-specific analytic modifications, which included reducing the number of diseases monitored using this method, allowing for customized signaling thresholds, and accounting for sudden changes in reporting (Table 2).

We reduced the ≈70 diseases to which $HLM_{original}$ had been applied to the 35 for which prospective and timely identification of clusters might result in public health action. For example, clusters of leprosy or Creutzfeldt-Jakob disease diagnoses within a 4-week period would not be informative because these diseases have long incubation periods, measured in years. We also excluded diseases that occur very infrequently or are nonexistent (defined as having an annual mean of <4 cases during 2008–2012). For example, we excluded tularemia and human rabies because any clusters of these diseases would be detected without automated analyses and because the underlying normality assumption of the method is violated for rare events.

Signals were most common at the neighborhood geographic level because of the increased noise resulting from small counts. Therefore, we also provided the option to reviewers to require >3 confirmed, probable, suspected, or pending cases to qualify as a signal at this geographic resolution.

Evaluation of $HLM_{refined}$

BCD implemented $HLM_{refined}$ on May 20, 2013, including automatically generating reports for disease reviewers to summarize information about cases included in signals (online Technical Appendix). To determine the effects of the

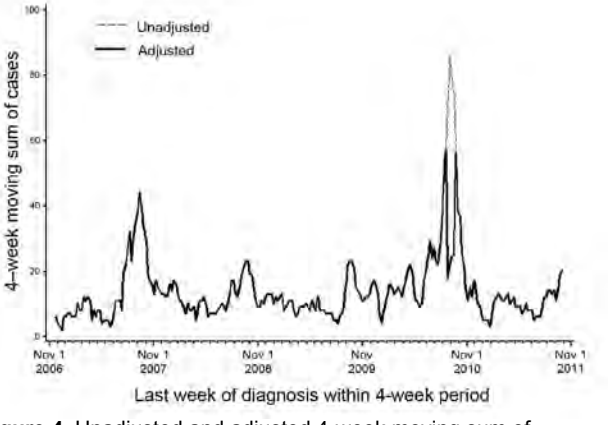

Figure 4. Unadjusted and adjusted 4-week moving sum of citywide dengue fever cases to illustrate adjustment for outliers in historical data, New York City, New York, USA, November 2006–October 2011.

above refinements, we compared signals detected during the 12 weeks after implementation with those that would have been detected had $HLM_{original}$ still been in place. A signal was defined as any set of consecutive 4-week periods, permitting 1-week gaps, where the disease counts were above historical limits for either $HLM_{original}$ or $HLM_{refined}$. Signals that were repeated in the same geographic area over multiple consecutive weeks were counted only once. Restricting analysis to a common set of 35 diseases (Table 2), we quantified the number of signals, determined the cause of any differences in signals between $HLM_{original}$ and $HLM_{refined}$, and monitored the outcome of any public health investigations triggered by automated signals.

We describe our experience with these methods in a government setting to support applied public health practice. In this setting, a complete list of true disease clusters and the resources to thoroughly investigate every statistical signal do not exist. We instead defined the set of true disease clusters as those identified using either method that could not be explained by any known systematic bias. We calculated type I and type II error rates using this set. Although artificial surveillance data generated through simulations have been created (*24*,*25*), those existing data do not reflect the dynamism and variability in actual reportable disease surveillance data, such as pending case reclassification (bias 1) and data accrual lags (bias 4). Accounting for this dynamism is essential for a valid comparison of $HLM_{original}$ and $HLM_{refined}$. Thus, we chose a practical and descriptive approach to evaluating these methods rather than a quantitative simulation study.

Table 2. Diseases included in analyses using $HLM_{refined}$ and details of customizations, New York City, New York, USA, May 20–August 5, 2013*

Disease	Minimum no. cases in UHF neighborhood to qualify for signal	Further customization
Amebiasis	5	
Anaplasmosis (human granulocytic)	3	
Babesiosis	3	
Campylobacteriosis	8	
Cholera	3	
Cryptosporidiosis	5	
Cyclosporiasis	3	
Dengue	3	
Ehrlichiosis (human monocytic)	3	
Giardiasis	5	
Haemophilus influenzae disease, invasive	3	
Hemolytic uremic syndrome	3	
Hepatitis A	5	
Hepatitis B (acute)	2†	
Hepatitis D	2†	
Hepatitis E	2†	
Legionellosis	5	
Listeriosis	3	
Malaria	3	
Meningitis, bacterial	4	
Meningitis, viral (aseptic)	3	
Meningococcal disease (*Neisseria meningitidis*)	3	
Paratyphoid fever	3	
Rickettisalpox	3	
Rocky Mountain spotted fever	3	Restrict analysis to confirmed, probable, and suspected cases and implement a 4-wk lag to allow for data accrual
Shiga toxin–producing *Escherichia coli* (including *E. coli* O157:H7) infection	3	
Shigellosis	10	
Staphylococcus aureus infection, vancomycin intermediate	3	
Streptococcus (group A) disease, invasive	5	Restrict analysis to confirmed, probable, suspected, and pending cases
Streptococcus (group B) disease, invasive	5	
Streptococcus pneumoniae disease, invasive	5	
Typhoid fever	3	
Vibrio spp. infection, noncholera (including *parahaemolyticus* and *vulnificus*)	3	
West Nile disease	3	
Yersiniosis	3	

* $HLM_{refined}$, refined method applied starting May 20, 2013; UHF, United Hospital Fund.
†These are the only diseases for which the signaling threshold was decreased below 3 cases.

Results

In the first 12 weekly analyses, $HLM_{original}$ would have produced 134 signals, and $HLM_{refined}$ produced 74 signals, a 45% decrease (Table 3). Of the $HLM_{original}$ signals during this period, 47 (35%) would have been at the neighborhood geographic resolution with fewer cases than the reviewers' threshold for action; these signals were omitted from further evaluation. Of the remaining 107 signals across both methods, 54 (50%) were detected by both methods, 33 (31%) only by $HLM_{original}$, and 20 (19%) only by $HLM_{refined}$.

We classified each signal into 1 of 3 categories (Table 4): attributable to an uncorrected bias toward signaling, attributable to the correction of a bias against signaling, or not attributable to any known systematic bias. Of the signals detected by $HLM_{original}$, 2 campylobacteriosis signals and 1 invasive *Haemophilus influenzae* disease signal were attributable to a bias toward signaling caused by an increasing trend in historical data. $HLM_{refined}$ missed 9 signals that were detected only by $HLM_{original}$ because the confirmatory proportion was larger in current data than in historical data.

Two signals detected by $HLM_{refined}$ were attributable to the removal of outliers from historical data; a legionellosis increase in the Bronx was masked by a prior increase in comparable weeks in 2009, and an amebiasis signal in a neighborhood was masked by a prior increase in comparable weeks in 2012. One signal detected by $HLM_{refined}$ was attributable to the adjustment of a decreasing trend in baseline disease counts of viral meningitis. Seventeen signals detected only by $HLM_{refined}$ were attributable to accounting for lags in data accrual (10 signals were first detectable after 1-week lag, 4 signals after 2 weeks, 2 signals after 3 weeks, and 1 signal after 4 weeks).

Overall, we identified 83 true clusters that could not be explained by any known systematic bias (i.e., 54 clusters identified by both $HLM_{original}$ and $HLM_{refined}$ and 29 clusters detected by only 1 of the methods and attributable to the correction of a bias against signaling). During the evaluation period, the percentage of all signals that did not correspond to these true clusters (type I error rate) for $HLM_{original}$ was 28% (24 of 87 signals) and, for $HLM_{refined}$, 0% (0 of 74 signals). The percentage of all true clusters that were not detected (type II error rate) for $HLM_{original}$ was 24% (20 of 83 true clusters) and, for $HLM_{refined}$, 11% (9 of 83 true clusters).

During these 12 weeks, 2 disease clusters occurred that we would have expected to detect using HLM. The first cluster of interest was a citywide increase in legionellosis in June 2013 (*26*). $HLM_{refined}$ first detected this increase with a cluster in Queens on June 24, 2013. The next week, both $HLM_{refined}$ and $HLM_{original}$ detected the citywide increase. Although $HLM_{refined}$ and $HLM_{original}$ might detect similar disease clusters at slightly different times because of differences in event inclusion criteria, the refinements do not directly affect timeliness.

On June 24, 2013, $HLM_{original}$ would have generated 16 automated signals (including 3 for campylobacteriosis), and $HLM_{refined}$ generated 5 signals (including 1 for campylobacteriosis); both methods detected a cluster of 11 campylobacteriosis cases in 1 neighborhood. After investigation, 8 of the cases were determined to be among children 0–5 years of age from Mandarin- or Cantonese-speaking families, 5 of whom had direct links to 1 of 2 local live-poultry markets. Consequently, pediatricians were educated about the association between live-poultry markets and campylobacteriosis, and health education materials about proper poultry preparation and hygiene were distributed to live-poultry markets.

Discussion

In refining the HLM to correct for major biases, we improved the ability to prospectively detect clusters of reportable infectious disease in NYC while preserving the simplicity of the output. Specifically, we addressed data challenges that are common to many jurisdictions, including improving consistency of case inclusion criteria, accounting for gradual trends and aberrations in historical data, and accounting for reporting delays.

$HLM_{refined}$ found fewer signals overall than $HLM_{original}$, which, in practice, is perhaps the greatest improvement. Disease reviewers had become accustomed to a large number of signals that did not represent true outbreaks, which led to dismissal of many signals without investigation. Fewer, higher quality signals produced by $HLM_{refined}$, supported by improvements in the ad hoc type I and type II error rates, led to more careful inspection and a higher probability of identifying true clusters, e.g., the true campylobacteriosis cluster in a Brooklyn neighborhood.

Although we consider $HLM_{refined}$ to be a substantial improvement upon $HLM_{original}$, we are aware that some limitations exist. In expanding case inclusion criteria to encompass all reports, we corrected a large bias but might have introduced a small bias. Because $HLM_{refined}$ considers the overall volume of reported cases, the implicit assumption is that the confirmatory proportion is constant over time outside of seasonal patterns. If this assumption is violated, and the confirmatory proportion differs between historical and current data, $HLM_{refined}$ can be biased. This bias is the reason that 9 signals detected by $HLM_{original}$ were not also detected by $HLM_{refined}$ during the evaluation period. Because these 9 signals might reflect disease clusters that would have been missed because of changes in the confirmatory proportion over time, we recommend implementing a lagged analysis that is restricted to confirmed, probable, and suspected cases. The signals produced by this lagged analysis can then be compared with signals produced in near real-time using all case statuses, and thus whether $HLM_{refined}$ systematically

Table 3. Geographic resolution of signals produced by $HLM_{original}$ and $HLM_{refined}$ in 12 weekly analyses, New York City, New York, USA, May 20–August 5, 2013*

Geographic area	≥3 Cases required for signal: No. signals produced by $HLM_{original}$	No. signals produced by $HLM_{original}$†	No. signals produced by $HLM_{refined}$†
City	14	14	8
Borough	40	40	26
UHF neighborhood	80	33	40
Total	134	87	74

*HLM, historical limits method; $HLM_{original}$, method as originally applied in NYC prior to May 20, 2013; $HLM_{refined}$, refined method applied starting May 20, 2013; UHF, United Hospital Fund.
†At neighborhood level, reviewer could require >3 cases for signal.

fails to detect clusters can be assessed. Implementing this approach post hoc yielded 2 additional clusters that both $HLM_{refined}$ and $HLM_{original}$ missed. Also, as with any method that defines geographic location according to patient residence, $HLM_{refined}$ can miss point source outbreaks when exposure occurs outside the residential area.

Next steps include addressing the arbitrary temporal and geographic units of analysis. $HLM_{refined}$ is optimized to detect clusters of 4-week duration at citywide, borough, or neighborhood geographic resolution. This method is likely to fail to detect clusters of shorter or longer duration, at sub-neighborhood geographic resolution, and in locations that span borough or neighborhood borders. In February 2014, we began applying the prospective space–time permutation scan statistic so we could use flexible spatial and temporal windows (*27*). We plan to expand the application of $HLM_{refined}$ to disease subspecies and serogroups within diseases (e.g., for salmonellosis) as this information becomes available in BCD's database system.

Health departments that receive a high volume of reports might consider adopting a method similar to $HLM_{refined}$ to improve prospective outbreak detection and contribute to timely health interventions. Simulation studies using complex artificial data that adequately reflect the dynamic nature of real-time surveillance data across a wide range of reportable diseases with variable trends over time and historical outbreaks would be valuable.

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We thank the members of the analytic team who work to detect disease clusters each week, including Ana Maria Fireteanu, Deborah Kapell, and Stanley Wang. We also thank Nimi Kadar who contributed substantially to the original SAS code for this method.

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Table 4. Explanation of signals produced by $HLM_{original}$ and $HLM_{refined}$ in the 12 weekly analyses, New York City, New York, USA, May 20–August 5, 2013*

Explanation	No. signals produced by $HLM_{original}$	No. signals produced by $HLM_{refined}$
Attributable to an uncorrected bias toward signaling		
Neighborhood disease count threshold too low	47†	0
Pending cases in current period	21	0
Increasing trends in baseline period	3	0
Total signals attributable to an uncorrected bias toward signaling	71	0
Attributable to the correction of a bias against signaling		
Confirmatory proportion higher in current period than in baseline period	9	0
Accounted for data accrual lags	0	17
Deleted outliers in baseline period	0	2
Adjusted for decreasing trends in baseline period	0	1
Total signals attributable to the correction of a bias against signaling	9	20
Not attributable to any known systematic bias	54	54
Total signals	134	74

*HLM, historical limits method; $HLM_{original}$, method as originally applied in NYC prior to May 20, 2013; $HLM_{refined}$, refined method applied starting May 20, 2013.
†These were excluded from the calculation of type I and type II error rates.

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Naturally Acquired Antibodies against *Haemophilus influenzae* Type a in Aboriginal Adults, Canada

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In the post-*Haemophilus influenzae* type b (Hib) vaccine era that began in the 1980's, *H. influenzae* type a (Hia) emerged as a prominent cause of invasive disease in North American Aboriginal populations. To test whether a lack of naturally acquired antibodies may underlie increased rates of invasive Hia disease, we compared serum bactericidal activity against Hia and Hib and IgG and IgM against capsular polysaccharide between Canadian Aboriginal and non-Aboriginal healthy and immunocompromised adults. Both healthy and immunocompromised Aboriginal adults exhibited significantly higher bactericidal antibody titers against Hia than did non-Aboriginal adults (p = 0.042 and 0.045 respectively), with no difference in functional antibody activity against Hib. IgM concentrations against Hia were higher than IgG in most study groups; the inverse was true for antibody concentrations against Hib. Our results indicate that Aboriginal adults possess substantial serum bactericidal activity against Hia that is mostly due to IgM antibodies. The presence of sustained IgM against Hia suggests recent Hia exposure.

Haemophilus influenzae is a human-restricted gram-negative bacterial pathogen that causes serious infectious diseases, including meningitis, sepsis, and pneumonia. Some strains express a polysaccharide capsule, a principal virulence factor that protects bacteria from immune defenses, e.g., complement-dependent bacteriolysis. On the basis of the chemical structure of the capsular polysaccharides, *H. influenzae* are divided into 6 serotypes (a, b, c, d, e, and f), and unencapsulated strains lacking the *cap* gene are referred to as nontypeable (*1*). *H. influenzae* type b (Hib) is the most virulent serotype; *H. influenzae* type a (Hia) is the second most virulent (*2*). Before the development of Hib conjugate vaccines in the 1980s, Hib was a major cause of pediatric meningitis (*3*). Hib conjugate vaccines induce production of antibodies against capsular polysaccharide capable of bactericidal activity providing protection against invasive disease (*4*–*6*). Rates of invasive Hib disease have been reduced by >90% in all countries where Hib vaccination programs have been introduced (reviewed in [*7*,*8*]).

Vaccination against Hib does not offer protection against other *H. influenzae* serotypes; in the post-Hib vaccine era, non–type b strains have become important in the etiology of invasive *H. influenzae* disease (reviewed by [*7*]). The course and severity of invasive Hia disease closely resemble the conditions caused by Hib (*9*).

Although invasive Hia disease is rare in most of the world, it is prevalent in specific geographic areas including Northern and Western Canada, Alaska, and the southwestern United States; furthermore, the burden of disease falls almost exclusively on Indigenous peoples living within these regions (*10*–*12*). In the region of this study (Northern Ontario), invasive Hia disease was reported at rates of 7/100,000 in 2004–2008 and between 7.7–23.2/100,000 among children <5 years of age during 2002–2008 (*13*,*14*). Recent analysis of invasive *H. influenzae* disease, including Hia, in a population of Canada that included a large proportion of Aboriginal persons found that 54% of adult case-patients had some serious underlying medical conditions, such as chronic renal failure (CRF) (*13*). We hypothesized that a lack of naturally acquired antibodies against Hia may contribute to the higher rates of invasive Hia disease in this regional population. To test this hypothesis, we measured concentration of serum IgG and IgM against capsular polysaccharide and functional antibody activity against both Hia and Hib in healthy adults and patients of Aboriginal background with confirmed CRF. Measured antibodies in Aboriginal persons were compared to those present in serum samples from non-Aboriginal persons residing in the same region.

Materials and Methods

Study Subjects

We recruited a convenience sample of 70 Aboriginal and 70 non-Aboriginal healthy adults from the area surrounding Thunder Bay, Ontario, Canada. Health status was based upon self-assignment. There was no significant difference

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in age between the groups (Table 1). To study a population of immunocompromised adults, we determined that a sample size of 30 subjects/group would give a power of 85% with an α of 0.05 (2-sided *t* test) for detection of 2-fold difference in mean geometrical titers of serum bactericidal activity against Hia. Therefore, we recruited 30 Aboriginal and 30 non-Aboriginal CRF patients undergoing hemodialysis at the Renal Services, Thunder Bay Regional Health Sciences Centre. On the basis of their age, 30 Aboriginal and 30 non-Aboriginal persons were selected for the healthy comparison group from the 140 persons in the healthy adult cohort to achieve a mean age that did not differ statistically from each ethnically matched CRF patient group (Table 1). None of the CRF or healthy comparison groups had been vaccinated against Hib. Among the 140 persons in the healthy adult cohort, 138 had not been vaccinated (we were unable to confirm Hib vaccination status of the remaining 2 persons). After acquiring informed consent from participants, we obtained serum samples and stored them at –80°C before use. All Aboriginal persons participating in the study were registered as such with the Canadian government. Recruitment took place during September 2010–August 2012. This study was approved by the Thunder Bay Regional Health Sciences Centre and Lakehead University Research Ethics Boards.

Serum Bactericidal Assay

For the serum bactericidal assay (SBA), the strains used were Hia 08–191, isolated in 2008 from the blood of a 47-year-old Aboriginal man at Sioux Lookout, Ontario, Canada (*14*), and Hib strain 10–090, isolated in 2010 from the cerebrospinal fluid of a 1-year-old girl in Manitoba, Canada (provided by Dr. Tsang, the National Microbiology Laboratory, Winnipeg, Manitoba). The Hib strain had been previously used in SBA assays (*15*,*16*). The SBA was performed essentially as previously described for Hib (*16*–*18*), but in the Hia SBA, surviving bacteria were incubated for 17 h at 32°C to obtain clearly separated colonies for enumeration. The SBA titers were defined as the reciprocal serum dilution required to kill ≥50% of the initial bacterial inoculum as described by Rouphael et al. (*18*). The Hia SBA assay specificity had been previously determined to be 99.3% (*18*). Discontinuous titers below the lower detection limit of 8 were reported as 4 for statistical purposes.

ELISAs for IgM and IgG against Hib Polysaccharide

We determined serum polysaccharide IgG antibody concentrations against Hib using a *Haemophilus influenzae* type b IgG ELISA kit (IBL International, Hamburg, Germany) according to the manufacturer's instructions. For statistical analysis, concentrations below the lower limit of quantification were assigned half the lower limit of quantification. Serum IgM reactive against Hib polysaccharide was quantified as described (*15*). Serum IgG was depleted by mixing serum samples with IgG/RF stripper (The Binding Site, Birmingham, UK) (*15*). The standard curve was generated by using the reference serum FDA lot 1983 (*19*) after IgG depletion. The range of quantification was 0.017–3.5 μg/mL.

ELISA for IgG against Hia Polysaccharide

We developed the assay based on published methods with modifications (*20*). Following isolation, the polysaccharide was oxidized and conjugated to human serum albumin, then purified and characterized as described by Cox et al. (*21*). Hia polysaccharide conjugated to human serum albumin was coated into 96-well ELISA plates (final concentration 0.1 μg/well). As secondary antibody, horseradish peroxidase–conjugated mouse antibody against human IgG (Hybridoma Reagent Laboratory, Baltimore, MD, USA) was used in a 1:4,000 dilution. After the addition of Sure Blue TMB peroxidase substrate (Mandel Scientific, Guelph, Ontario, Canada) the colorimetric substrate was detected by using a microplate reader (BioTek Powerwave XS; Winooski, VT, USA) at 450 nm with 630 nm reference. Quantification of antibody was performed by using a previously described reference serum (4.1 μg/mL) as a standard (*20*). The quantification range was 0.10–4 μg/mL.

ELISA for IgM against Hia Polysaccharide

To quantify IgM against Hia polysaccharide, we used the protocol for IgG against Hia polysaccharide with modifications. After coating, the plates were blocked for 2 h at

Table 1. Demographic characteristics of Aboriginal and non-Aboriginal groups studied for antibodies against *Haemophilus influenzae* type a, Thunder Bay region, northwestern Ontario, Canada, 2010–2012*

Group	No.	Age, y				No. (%) female	No. (%) ≥60 y
		Mean	Median	SD	Range		
Aboriginal CRF patients	30	54	54	±13.4	29–78	21 (70)	10 (33)
Aboriginal healthy comparison group	30	49.1	47	±9.4	39–75	25 (83)	4 (13)
Non-Aboriginal CRF patients	30	60.3	64.5	±13.9	26–79	11 (37)	20 (67)
Non-Aboriginal healthy comparison group	30	58.7	56	±9.4	45–80	18 (60)	11 (37)
Healthy Aboriginal adults	70	37.1	33.5	±12.7	19–75	58 (83)	4 (6)
Healthy non-Aboriginal adults	70	41.3	43	±14.1	22–68	44 (63)	7 (10)

*CRF, chronic renal failure. No significant age difference between healthy Aboriginal and non-Aboriginal adults (p = 0.067), Aboriginal and non-Aboriginal CRF patients (p = 0.081), Aboriginal CRF patients and Aboriginal healthy comparison group (p = 0.11), non-Aboriginal CRF patients and non-Aboriginal healthy comparison group (p = 0.60), p<0.05 between Aboriginal and non-Aboriginal healthy comparison groups (p = 0.0002). Healthy comparison groups (n = 30) were drawn from the large group of healthy adults (n = 70).

room temperature with antibody dilution buffer containing 1% fish gelatin (Sigma-Aldrich, Oakville, Ontario, Canada). Horseradish peroxidase–conjugated goat IgM against human IgM (SouthernBiotech, Birmingham, AL, USA) diluted 1:5,000 was used as the secondary antibody. The concentration of IgM against Hia polysaccharide (3.84 μg/mL) in the standard was determined by cross-standardization (*22*) to the Hib standard (Food and Drug Administration, 1983), which has an assignment of 3.5 μg/mL for IgM against Hib polysaccharide (*23*). The range of quantification was 0.01–18 μg/mL; for statistical purposes, samples with values less than the lower limit of quantification were assigned a value 1/2 the lower limit.

Complement Activity

To rule out complement deficiency in study participants, we assessed the total classical complement pathway activity in serum samples using the CH_{50} Eq (Quidel, CA, USA) immunoassay according to the manufacture's protocol; results were expressed as CH_{50} equivalent units per mL.

Statistical Analysis

We performed transformation of $\log_{10}$ data before analysis. Statistical significance was assessed by conducting Student *t* test using Graph-Pad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). Geometric mean antibody concentrations (GMC), SBA geometric mean titers (GMT), and 95% CI were calculated for each group. The criterion of detectable SBA GMT was determined by using Fisher exact test; p values <0.05 were considered significant.

Results

Functional Antibody Activity Specific to Hia

Among a group of 140 healthy adults, Aboriginal persons had a significantly higher Hia SBA GMT, i.e., 351.4 (95% CI = 226.8–544.5) compared to the GMT of 182.8 (95% CI = 115.5–289.4) in the non-Aboriginal group (Figure). Similarly, Aboriginal CRF patients exhibited a significantly higher Hia SBA GMT than their non-Aboriginal counterparts, as shown in Table 2. There was a tendency toward lower Hia SBA GMTs in both Aboriginal and non-Aboriginal CRF-patient groups compared to their corresponding healthy comparison groups, but the differences were not statistically significant (Table 2).

Serum Polysaccharide IgM and IgG against Hia

Aboriginal CRF patients showed a tendency to have lower concentrations of IgM against Hia polysaccharide than the Aboriginal healthy comparison group, but the difference was not statistically significant (Table 2). No difference in IgM against Hia polysaccharide was found between Aboriginal and non-Aboriginal CRF patients, non-Aboriginal CRF patients and the non-Aboriginal healthy comparison group, or between all CRF patients and all healthy comparison groups. Likewise, no difference in IgG against Hia polysaccharide concentrations between the groups was detected (Table 2). The combined IgM GMC against Hia of all groups was significantly higher (p = 0.0013) than IgG GMC against Hia, i.e., 2.38 μg/mL (95% CI = 1.84–3.08) versus 1.44 μg/mL (95% CI = 1.22–1.70); the average ratio of IgM against Hia to IgG against Hia was 1.65:1.

Functional Antibody Activity Specific to Hib

No difference in serum bactericidal activity against Hib between Aboriginal and non-Aboriginal CRF patients was found. However, Aboriginal CRF patients had lower SBA GMT compared to the Aboriginal healthy comparison group. Overall, CRF patients had a lower SBA GMT than all healthy comparison groups (Table 2).

Serum Polysaccharide IgM and IgG against Hib

Aboriginal CRF patients had significantly lower concentrations of IgM and IgG against Hib polysaccharide than the Aboriginal healthy comparison group. However, no statistically significant difference in IgM or IgG against Hib polysaccharides was found between Aboriginal and non-Aboriginal CRF patients, or non-Aboriginal CRF patients and non-Aboriginal healthy comparison group. Overall, CRF patients had a significantly lower IgG GMC compared to all healthy comparison groups (Table 2). The combined IgM GMC against Hib of all groups was significantly lower (p<0.0001) than IgG GMC against Hib, i.e., 0.061 μg/mL (95% CI = 0.045–0.082) versus 0.85 μg/mL (95% CI = 0.68–1.08); the average ratio of IgM against Hib to IgG against Hib was 0.072.

Classical Complement Activity

The endogenous classical complement activity was measured in all study participants. The geometric means of all groups were in the range between 96.63 Eq U/mL (95% CI = 78.63–118.6) in the non-Aboriginal healthy comparison group and 118.9 Eq U/mL (95% CI = 99.13–142.5) in the Aboriginal healthy comparison group. The values were above the established cutoff point used to define abnormally low classical complement activity (<70 CH_{50} equivalent units per mL) according to manufacturer and did not statistically differ from each other (data not shown).

Discussion

Compared to the general population, North American Indigenous populations have endured higher rates of invasive *H. influenzae* disease in both the pre- and post-Hib vaccine era (*12*). Although in the pre-Hib vaccine era, Hib was the major cause of invasive *H. influenzae* disease, cases attributed to Hia were also reported among some indigenous

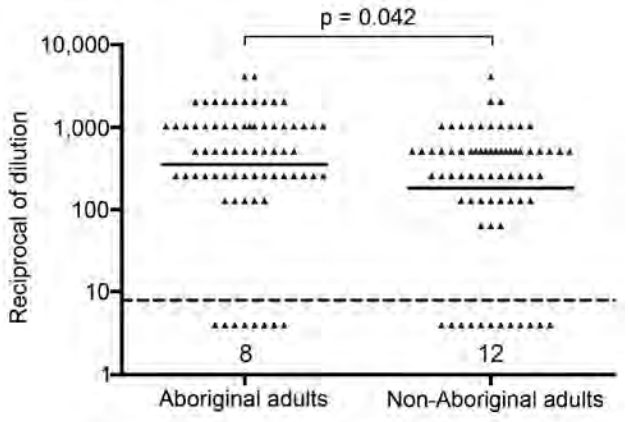

Figure. Antibody mediated bactericidal activity against *Haemophilus influenzae* type a in healthy Aboriginal (n = 70) and non-Aboriginal (n = 70) adults residing in the Thunder Bay region of northwestern Ontario, Canada, 2010–2012. The solid line indicates geometrical mean titer. The dashed line indicates the lower limit of detection; the number of individual samples below this limit is indicated on the graph.

populations; for example, Hia accounted for 17% of invasive *H. influenzae* cases in a White Mountain Apache Indian community (October 1981–January 1983) (*24*). In the post-Hib vaccine era, Hia has emerged as a prominent cause of invasive disease among Alaska Natives and Aboriginal peoples of Canada (*14*,*25*,*26*). In northwestern Ontario (Canada), Hia is the serotype that most often cause invasive *H. influenzae* disease among Aboriginal persons, with an incidence rate of 7/100,000 during 2004–2008 (*13*,*14*). Recent studies in Northern Canada and Alaska found much higher prevalence of invasive Hia disease among the Aboriginal population than among the non-Aboriginal population. In Alaska, 88% of the cases occurred in Alaska Native persons (2002–2011) (*25*); in the Canadian North, 91% of the cases occurred in Aboriginal persons; the remaining 9% listed no ethnicity (2000–2010) (*26*). The incidence rate of invasive Hia disease in Alaska Native children <5 years in 2002–2011 was 36 times higher than in non-Native children (*25*). The factors related to an increased incidence of invasive Hia disease in Aboriginal populations are obscure. In this study, we questioned whether there were differences in the naturally acquired antibodies against Hia between Aboriginal and non-Aboriginal healthy and immunocompromised adults living in the same geographic area.

Our study shows an increased functional activity of serum antibody against Hia in Aboriginal adults of Canada than in non-Aboriginal persons living in the same area, which is likely caused by a substantial quantity of IgM antibodies. The presence of elevated IgM concentrations relative to IgG concentrations that are specific to Hia is suggestive of recent exposure to this pathogen in this study population. This is more apparent when the IgM against Hib were found to be low (<0.08 μg/mL) in most of the study groups, except for the Aboriginal healthy comparison group. These low levels of Hib antibodies can be attributed to the successful implementation of Hib vaccination (88% mean vaccine coverage) in the Canadian pediatric population and the effect of herd immunity (*27*).

In both healthy comparison groups and immunocompromised adults, IgM against Hia concentrations were higher than those of IgG, although the ratio was opposite for IgM and IgG isotypes of antibodies to Hib. A prevalence of IgM in the polysaccharide antibody repertoire against Hia among unvaccinated adults further suggests that naturally acquired antibodies could be the result of exposure to some cross-reactive antigens (*28*). High concentrations of IgM against Hib polysaccharide have also been found in non-vaccinated Alaska Native adults and potentially linked to continuous Hib carriage in this population (*23*).

Among healthy adults, serum antibody in the Aboriginal group exhibited much higher functional activity against Hia than that of the non-Aboriginal group as determined by SBA (Figure). Our findings contradict a presumption that a high rate of invasive Hia disease among Aboriginal peoples is due to a lack of naturally acquired antibodies. However, in northwestern Ontario, all adult cases of invasive Hia infection occurred in persons who had some underlying conditions associated with secondary immunodeficiency, such as type 2 diabetes or chronic obstructive pulmonary disease (*13*). Therefore, healthy adults may poorly represent the susceptible groups.

CRF patients undergoing hemodialysis are immunocompromised as a consequence of conditions underlying their renal failure as well as the hemodialysis procedure (*29*). Our previous research demonstrated that this patient group has a low concentration of antibodies against Hib that may be insufficient to protect against invasive Hib disease (*15*,*16*). Of note, Aboriginal peoples of Canada have a higher prevalence of end-stage renal disease and acquire it at younger ages than the general population (*30*). The SBA GMT against Hib was significantly lower in CRF patients than in healthy comparison groups which is consistent with our previous data on Hib immunity among patients in CRF (*15*,*16*). In addition to the presence of specific antibodies, the complement system is essential for defense against infections caused by gram-negative pathogens (*31*). Encapsulated *H. influenzae* are cleared by type specific antibodies against capsular polysaccharides, which activate the classical complement pathway leading to bacteriolysis (*32*). Because CRF patients often suffer protein loss related to the nephrotic syndrome and dialysis, their serum bactericidal activity in vivo may be impaired because of decreased complement concentration and lower antibodies in circulation. Because the SBA protocol uses a standardized exogenous complement source, a complement deficiency in

Table 2. Concentrations of antibodies against *Haemophilus influenzae* type a and type b and serum bactericidal assay titers in Aboriginal and non-Aboriginal patients in chronic renal failure and comparison groups of healthy persons from the Thunder Bay region, northwestern Ontario, Canada, 2010–2012*

Variable	Age-matched comparison groups of healthy Aboriginal persons and those with CRF	Aboriginal CRF patients	Non-Aboriginal CRF patients	Non-Aboriginal healthy comparison group age-matched to non-Aboriginal CRF	Age-matched comparison group regardless of race	CRF patients regardless of race
No. patients	30	30	30	30	60	60
Hia SBA GMT	268.1	147.0	49.64	104.0	167.0	85.43
95% CI	132.8–541.4	72.9–296.4	21.7–113.3	49.2–219.7	100–278.9	49.6–147.2
p value†	p = 0.22	**p = 0.045**	p = 0.18		p = 0.076	
IgM against Hia, GMC	3.75	1.94	2.60	1.69	2.52	2.25
95% CI	2.28–6.15	1.08–3.50	1.69–4.01	0.95–3.01	1.72–3.69	1.57–3.21
p value	p = 0.086	p = 0.42	p = 0.23		p = 0.66	
IgG against Hia, GMC	1.81	1.56	1.20	1.27	1.52	1.37
95% CI	1.26–2.60	1.12–2.17	0.89–1.63	0.91–1.77	1.19–1.93	1.10–1.71
p value	p = 0.53	p = 0.25	p = 0.81		p = 0.54	
Hib SBA GMT	181.0	23.7	15.28	25.4	67.81	19.03
95% CI	84.0–390.2	11.09–50.62	6.88–33.94	11.26–57.30	37.21–123.6	11.12–32.56
P value	**p = 0.0003**	p = 0.42	p = 0.37		**p = 0.002**	
IgM against Hib, GMC	0.11	0.04	0.08	0.04	0.07	0.06
95% CI	0.069–0.18	0.02–0.079	0.05–0.12	0.02–0.08	0.04–0.10	0.04–0.08
p value	**p = 0.015**	p = 0.10	p = 0.089		p = 0.64	
IgG against Hib, GMC	1.63	0.61	0.62	0.87	1.19	0.61
95% CI	1.06–2.51	0.39–0.95	0.34–1.11	0.61–1.24	0.91–1.58	0.43–0.88
p value	**p = 0.0018**	p = 0.95	p = 0.31		**p = 0.004**	

*CRF, chronic renal failure; Hia *Haemophilus influenzae* type a; SBA, serum bactericidal assay; GMT, geometric mean titer; GMC, geometric mean concentration (μg/mL); CI, confidence interval; Hib, *Haemophilus influenza*e type b.
†p values (Student *t* test [2-sided after log transformation]) are provided between each pairwise comparison. Significance level set at p<0.05; bold text indicates statistically significant p values.

tested serum samples would not influence the assay results. However, to rule out complement deficiency as a factor underlying susceptibility to invasive Hia disease, we tested the total classical complement activity in serum samples of study participants and found this parameter within the normal range in all the groups.

Although higher bactericidal activity against Hia was found in Aboriginal CRF patients than in non-Aboriginal ones, no significant difference in SBA GMT against Hib was detected between these groups. Despite major biological similarities between Hia and Hib, these 2 organisms have different natural histories because current vaccination programs cover Hib but not Hia. Pediatric Hib vaccination leads to a decreased carriage rate of Hib among adults by herd immunity; this may cause a decrease in natural boosting of antibody responses in nonvaccinated populations (*33*,*34*). Since there is no vaccine pressure on Hia, the difference in functional antibody activity between Aboriginal and non-Aboriginal adults may hypothetically depend on higher exposure to Hia in Aboriginal communities than in the general population. In addition, exposure to cross-reactive antigens of other bacteria, such as *Streptococcus pneumoniae* serotype 6B or *Bacillus pumilus* Sh 18 may potentially stimulate production of antibodies to Hia (*35*,*36*). Persistent circulation of *S. pneumoniae* in Aboriginal communities has been documented that may contribute to the development of natural immunity against Hia in Aboriginal adults (*37*). A review of the literature indicated only 1 other report of IgG measurements specific to Hia. Schmidt et al. analyzed an antibody against Hia in the cord blood of healthy neonates in Mexico and Chile; concentrations of IgG against Hia found in that study were 10- to 30-fold lower than the IgG against Hia in the CRF patients in this study (*20*). Because the predominant isotype of antibody against capsular polysaccharide is IgG2, which does not efficiently cross the placenta (*38*), analysis of neonatal cord blood samples may underestimate the quantity of antibody present in the maternal peripheral blood; cord blood would not include maternal IgM.

Our study has several limitations. In general, Aboriginal CRF patients undergoing hemodialysis are younger than their non-Aboriginal counterparts (*39*), and this was also true for our study groups. Therefore, a substantial age difference between the Aboriginal and non-Aboriginal healthy comparison groups precluded direct comparisons between the CRF groups and the large cohort groups. We compared each ethnic group to its own healthy comparison group to eliminate a possible effect of age on antibody concentrations and functional activity. Because children exhibit the majority of invasive Hia disease cases in our study region and elsewhere (*13*,*25*,*26*,*40*), the demographics of the present study do not fully reflect the immunoepidemiology of this infection. Future work should address natural immunity against Hia in the pediatric population. It will be important to study Hia antibody concentrations in neonatal cord blood serum samples in our population,

as transplacentally acquired IgG may confer protection against invasive Hia disease in a similar manner to what is observed for Hib (*5*). Because the correlates of protection against invasive Hia disease have not yet been defined, the clinical interpretation of our findings is limited. Moreover, because of the sampling methods, this study may not represent other groups of North American indigenous peoples because they are highly genetically and phenotypically diverse and live in various environments. Extending the study of seroepidemiology of Hia infection will be critical to clarifying the reasons behind an increased susceptibility to Hia among certain population groups.

Although we did not conduct colonization studies, our results suggest that the high anti-Hia SBA titers in Aboriginal peoples are likely the result of a higher rate of Hia circulation within Aboriginal communities. It is crucial to study Hia colonization rates in this population to determine whether these natural antibodies are due to Hia exposure or exposure to cross-reacting bacterial species.

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Infectious Causes of Encephalitis and Meningoencephalitis in Thailand, 2003–2005

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Learning Objectives

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

- Distinguish the prevalence of abnormal ancillary testing results among patients with encephalitis
- Assess demographic and clinical characteristics of patients with encephalitis
- Discuss the rate of confirmed or probable etiology of encephalitis in the current study
- Identify the most common causative organism associated with encephalitis in the current study.

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Acute encephalitis is a severe neurologic syndrome. Determining etiology from among ≈100 possible agents is difficult. To identify infectious etiologies of encephalitis in Thailand, we conducted surveillance in 7 hospitals during July 2003–August 2005 and selected patients with acute onset of brain dysfunction with fever or hypothermia and with abnormalities seen on neuroimages or electroencephalograms or with cerebrospinal fluid pleocytosis. Blood and cerebrospinal fluid were tested for >30 pathogens. Among 149 case-patients, median age was 12 (range 0–83) years, 84 (56%) were male, and 15 (10%) died. Etiology was confirmed or probable for 54 (36%) and possible or unknown for 95 (64%). Among confirmed or probable etiologies, the leading pathogens were Japanese encephalitis virus, enteroviruses, and *Orientia tsutsugamushi*. No samples were positive for chikungunya, Nipah, or West Nile viruses; *Bartonella henselae*; or malaria parasites. Although a broad range of infectious agents was identified, the etiology of most cases remains unknown.

Acute encephalitis is a severe neurologic syndrome that is often associated with substantial illness and death. It can be caused by any of ≈100 infectious agents that vary by geographic region. Among leading known causes of encephalitis in the United States and worldwide are arthropodborne viruses (arboviruses) and herpesviruses (*1–9*). Data on encephalitis in Southeast Asia are limited, but previous studies have identified Japanese encephalitis virus (JEV) and herpesviruses as common causes (*10–12*). Dengue virus has also been associated with encephalopathy and other neurologic findings (*4*,*13–15*). Over the past decade, several viral encephalitides have emerged in Southeast Asia, including Nipah virus and enterovirus 71, and led to unexpected outbreaks of neurologic disease (*4*,*16–23*).

Determining the etiology of encephalitis is difficult. The definition varies, and distinguishing the neurologic syndrome of encephalitis from meningoencephalitis or even meningitis or encephalopathy can be challenging (*24*,*25*). In recent studies, the etiology for most cases was not determined despite extensive testing (*1*,*6–12*,*26*). One characteristic of past studies is that laboratory diagnosis for encephalitis was often not complete because of lack of available diagnostics, limited scope of pathogens studied, or difficulty obtaining adequate specimens. Detection capabilities are limited by testing for only a limited core battery of pathogens or the most likely pathogens on the basis of exposure history and clinical information and by use of conventional diagnostics.

To determine the spectrum of encephalitis etiologic agents in Thailand, we conducted a prospective study and used an expanded testing approach. We used a case definition consistent with definitions used in prior studies, requiring acute brain dysfunction and evidence of inflammation and including patients who also had meningeal inflammation with an encephalitic component (meningoencephalitis) (*1*,*10*,*12*). We sought to identify etiologic pathogens for patients with a clinical syndrome consistent with encephalitis and meningoencephalitis and to describe the clinical features and outcomes associated with different causes.

Methods

Study Sites

Patients were recruited from 7 hospitals in Thailand: 5 in Bangkok (Queen Sirikit National Institute of Child Health, Rajvithi Hospital, Prasat Neurologic Institute, Ramathibodi Hospital, and Phramongkutkao Hospital) and 2 in the southern city of Hat Yai (Hat Yai Hospital, Prince Songkhla University Hospital). The main study physicians in each hospital were specialists in neurology or infectious diseases. During July 2003–August 2005, physicians identified potential study participants who met eligibility criteria and referred them to a study nurse, who obtained written informed consent. The protocol was approved by an institutional review board at the Centers for Disease Control and Prevention (CDC) and the Thailand Ministry of Public Health.

Enrollment Criteria and Case Definition

Enrollment criteria for patients of any age included all of the following: 1) acute brain dysfunction requiring hospitalization (new encephalopathy [i.e., altered mental status with or without lethargy], new onset of diffuse or focal central neurologic findings, or new onset of seizures); 2) acute onset of brain dysfunction within 14 days before or 7 days after admission to a study hospital; 3) documented fever (≥38°C), history of fever, or hypothermia (≤35°C); and 4) clinical indication for lumbar puncture as determined by the patient's physician. After enrollment, participants were required to meet at least 1 of the following 3 criteria to meet the case definition of acute encephalitis or meningoencephalitis: 1) abnormal findings consistent with encephalitis seen on neuroimages obtained by computed tomography (CT) scan, magnetic resonance imaging (MRI), or cranial ultrasonography; 2) abnormal findings on electroencephalogram (EEG) consistent with encephalitis; or 3) cerebrospinal fluid (CSF) pleocytosis (≥15 leukocytes/mm^3 for infants ≤6 weeks of age and ≥5 leukocytes/mm^3 for patients >6 weeks of age). Patients found to have an alternative confirmed diagnosis before discharge that explained their signs and symptoms (e.g., metabolic encephalopathy) were excluded from further study.

Epidemiologic Data and Specimen Collection

For each patient, study physicians completed standardized admission and discharge surveys documenting medical

history, signs and symptoms, and neuroimaging and EEG results. Research nurses completed an extensive questionnaire about demographics, medical and exposure history, and laboratory results. Study nurses completed a follow-up questionnaire during each patient's convalescent-phase visit 3–6 weeks after enrollment. The following specimens were collected: CSF (up to 6.5 mL); acute- and convalescent-phase blood (12.5–22.5 mL from children <5 years of age and 25.5 mL from all others); and oropharyngeal swab, saliva (0.7 mL), urine (10 mL), and fecal (10–20 g) specimens.

Specimen Handling, Storage, and Testing

Specimens were tested for the presence of >30 pathogens (Table 1, http://wwwnc.cdc.gov/EID/article/21/2/14-0291-T1.htm). After collection, specimens were immediately separated into portions for clinical testing at hospitals and research testing at reference laboratories (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/2/14-0291-Techapp1.pdf). Within 24 hours of collection, specimens destined for reference laboratories were aliquoted for distribution to >20 laboratories at the Thailand National Institute of Health and CDC. Any specimens that could not be aliquoted within 24 hours were stored at –20°C and then at –70°C after aliquoting. Specimens from Hat Yai were transported on dry ice to Bangkok every 2–4 weeks. If CSF or serum sample volume was limited, the order of specimen testing was prioritized (online Technical Appendix).

Etiologic Classification

Definitions were created to define the etiologic link between identified pathogens and encephalitis; the etiology for each case was classified as confirmed, probable, possible, or unknown (Table 1). Each case could be assigned >1 etiology. The classification system considered the nature of an agent (well-established cause of encephalitis or not) and etiologic significance of a given positive laboratory test result.

Etiology was considered confirmed for cases with positive results for PCR, culture, antigen, or pathogen-specific IgM in CSF for a pathogen considered to be a well-established cause of encephalitis. For arboviruses commonly associated with encephalitis (i.e., JEV or West Nile virus), documentation of acute infection in paired serum samples was considered the diagnostic standard and therefore was considered confirmatory.

Etiology was considered probable for cases with positive results that were strongly suggestive but not considered confirmatory and not clearly established as diagnostic for encephalitis in all situations or cases for which a pathogen not generally established as a cause of encephalitis was detected in CSF. For example, etiology would be considered probable if PCR of CSF was positive for lymphotropic agents (e.g., cytomegalovirus, Epstein-Barr virus [EBV]) or if serum testing results provided evidence of acute infection for pathogens known to be associated with encephalitis, such as *Orientia tsutsugamushi*, *Bartonella henselae*, and *Mycoplasma pneumoniae*.

Etiology was considered possible for cases with no laboratory evidence of CNS involvement but some evidence of acute infection that suggested a potential etiologic role in encephalitis, such as a ≥4-fold rise in antibody titer for enteroviruses or an oropharyngeal swab sample positive for influenza or parainfluenza viruses. In addition, etiology was considered possible for cases for which information to determine etiology of encephalitis (e.g., human adenoviruses 40 and 41) was insufficient, even if detected in CSF (*27*). Etiology was considered unknown for cases for which all testing results were negative.

In an effort to differentiate between cases of encephalitis and meningoencephalitis, we defined a subset of patients who met the case definition and specifically had CSF pleocytosis and stiff neck as having meningoencephalitis. To provide a complete description of all patients who met the case definition, we retained the 8 patients for whom a sole confirmed bacterial agent commonly associated with bacterial meningitis was found. Pertinent analyses were performed with and without these 8 patients. For 1 patient for whom a bacterial etiology was confirmed, a viral etiology (JEV) was also confirmed; thus, this patient was not excluded from either analysis.

Statistical Analyses

We present descriptive data with case counts and frequencies. We used the Wilcoxon rank-sum test to compare continuous variables between the confirmed/probable and possible/unknown etiologic groups. The prevalence of categorical variables was compared by using χ^2 analysis or the Fisher exact test. We considered 2-sided p values <0.05 to be statistically significant. Statistical analyses were performed by using SPSS Statistics for Windows version 21.0 (IBM Corp., Armonk, NY, USA).

Results

We enrolled 193 patients, among whom 149 (77%) met the case definition for acute encephalitis (Figure 1). Of the 149 acute encephalitis patients, CSF pleocytosis was found for 125 (84%), abnormal neuroimages for 80 (54%) (45 brain MRI, 55 brain CT, 6 cranial ultrasonography), and EEG findings consistent with encephalitis (categories not mutually exclusive) for 28 (19%). Of the 149 patients, 84 (56%) were male and median age was 12 (range 0–83) years. A median of 5 patients were admitted each month, varying somewhat by season (Figure 2). A total of 30 (20%) patients were from Songkhla; 73 (49%) reported having lived in a city or town in the past 3 months. Median time between onset of neurologic symptoms and admission was 1

day (range 12 days before admission to 7 days after), and median length of hospital stay was 15 (range 1–180) days. A total of 22 (15%) patients had an underlying condition (e.g., HIV infection, malignancy, diabetes mellitus). Of 3 main antimicrobial treatments given empirically during hospitalization, acyclovir was given to 62 (42%) patients; a third- or fourth-generation cephalosporin to 97 (65%), 16 of whom also received a carbapenem; doxycycline to 10 (7%); at least 1 of these drugs to 122 (82%); and all 3 drugs to 4 (3%). A total of 15 (10%) patients died.

With regard to hospital testing, blood culture was performed for 129 (87%), CSF culture for 141 (95%), and both cultures for 123 (83%). Growth occurred on 21 (16%) blood cultures and 19 (13%) CSF cultures (Table 2); pathogens grew on both cultures for 7 patients. No patients had positive malaria testing results, and 8 (5.4%) had positive HIV results.

For most patients, specimens were available for additional testing at the reference laboratories (Thailand National Institute of Health and CDC). CSF specimens were available for 147 (99%) (median 3.1, range 0.8–13 mL), acute-phase serum for 145 (97%) and convalescent-phase serum for 129 (87%) (median time between sample collection 24 [range 14–39] days), oropharyngeal swab samples for 141 (95%), saliva for 138 (93%), feces for 119 (80%), and urine for 143 (96%) patients.

Case Designation by Etiologic Classification

Of the 149 cases, etiology was confirmed for 37 (25%), probable for 17 (11%), possible for 44 (30%), and unknown for 51 (34%) (Table 3). Among confirmed etiologies, JEV was identified in 15 patients and dengue virus in 2. In >1 patient, infection with the following was also identified: enteroviruses (n = 6), *Cryptococcus* spp. (n = 3), *Haemophilus influenzae* (n = 3), *Streptococcus pneumoniae* (n = 3), and varicella-zoster virus (VZV; n = 2). Among case-patients whose illness met the definition for confirmed etiology, a bacterial pathogen commonly associated with meningitis was confirmed for 8 patients (*H. influenzae* for 3, *S. pneumoniae* for 3, *Neisseria meningitidis* for 1, and *Escherichia coli* for 1).

Among patients for whom etiology was probable, *O. tsutsugamushi*, which causes scrub typhus, was found in 6 patients (5 became ill while in central Thailand and were hospitalized in Bangkok); JEV in 6, EBV in 3 (concurrent with *Cryptococcus* spp. in 1 and VZV in 1); *M. pneumoniae* in 3; and *Rickettsia conorii*, which causes spotted fever, in 3 patients. Among the 54 patients for whom etiology was confirmed/probable, a potentially treatable pathogen was identified for 25 (46%) (*C. neoformans*, *E. coli*, herpes simplex virus, *H. influenzae*, influenza viruses, *M. pneumoniae*, *N. meningitidis*, *O. tsutsugamushi*, *R. conorii*, *S. pneumoniae*, and *Treponema pallidum*). We did not include the patient with confirmed JEV and *Salmonella* group D infection in the treatable category.

Among the 98 patients for whom at least 1 pathogen was identified, a total of 156 pathogens were detected (Table 3); of 40 (41%) patients for whom >1 pathogen was detected, etiology was classified as possible for most. Of the 54 patients for whom etiology was confirmed/probable, >1 pathogen was classified as confirmed/probable for 6 (11%): (JEV/*O. tsutsugamushi*/*R. conorii*, JEV/*R. conorii*, JEV/*Salmonella*, *Cryptococcus* spp./EBV, VZV/EBV, VZV/influenza virus), whereas at least 1 possible etiology was also detected for 28 (52%) ($p<0.01$). Of 44 patients for whom a possible etiology was identified, >1 possible pathogen was detected for 12 (27%).

Of the 149 patients, 68 (46%) met the definition for meningoencephalitis; for 38 (56%) of these patients, EEG or neuroimaging findings were abnormal, consistent with encephalitis. Of these 38 patients, pathogens considered confirmed/probable etiologic agents were detected for 12

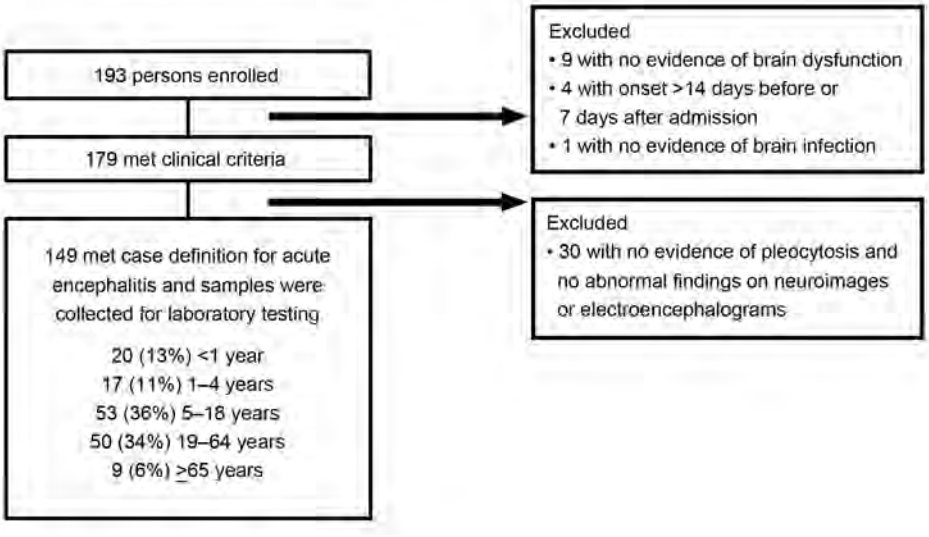

Figure 1. Schematic of enrolled patients who met case definition for inclusion in study of patients with encephalitis, Thailand, 2003–2005.

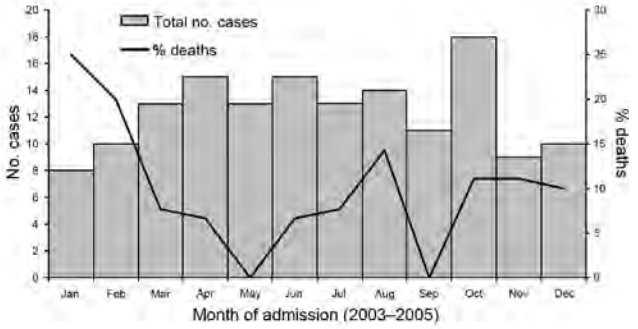

Figure 2. Month of admission for 149 patients with encephalitis, Thailand, 2003–2005.

(32%): JEV for 8 (also *R. conorii* for 2 and *O. tsutsugamushi* for 1), *Cryptococcus* for 2, *M. pneumoniae* for 1, and *R. conorii* for 1. Of the 30 remaining patients, pathogens that conferred a confirmed/probable etiologic classification were detected for 16 (53%): JEV for 6, *O. tsutsugamushi* for 4, enterovirus for 3, *N. meningitidis* for 1, herpes simplex virus 1/2 (not distinguished) for 1, and VZV/EBV (positive for both) for 1.

At admission, very few demographic and clinical differences between those with confirmed/probable and possible/unknown etiologies were found (Table 4). Among patients for whom etiology was confirmed/probable, median temperature was higher, loss of consciousness was less common, and stiff neck was more common. When the 8 patients with bacterial meningitis were excluded from analysis, patients for whom etiology was confirmed/probable had a higher median temperature than those for whom etiology was possible/unknown (37.9 vs. 37.7, p = 0.04) and were more likely to have a stiff neck (54% vs. 31%, p = 0.01). Among the 54 patients for whom etiology was confirmed or probable, no differences were found for demographic characteristics and only 1 difference was found for clinical characteristics at admission (Table 4) between those for whom etiology was or was not treatable; temperature was lower among those for whom etiology was treatable (37.9 vs. 39, p<0.01).

Outcomes

A total of 15 (10%) patients died. The mortality rate was lower, but not significantly, among patients for whom etiology was confirmed/probable than for those for whom etiology was possible/unknown (3.9% vs. 14%, p = 0.09; Table 4). When the 8 patients for whom only a confirmed bacterial etiology was found were excluded from analysis, this difference was similar (4.3% vs. 14%, p = 0.14). Among those who died, etiology was confirmed for 2 (*Cryptococcus* spp. in a 5-year-old boy and VZV in a 51-year-old HIV-infected woman). Among those who died, 9 (60%) were male and the median age was 34 years of age (1 was <1 year, 1 was 1–4 years, 4 were 5–18 years, 8 were 19–64 years, and 1 was ≥65 years). The median interval between hospital admission and death (or discharge for survivors) was 6 days (vs. 16 days to discharge, p = 0.3), between onset of neurologic symptoms and death was 10 days (vs. 18.5 days to discharge, p = 0.3), and between fever onset and death was 10 days (vs. 20 to discharge, p = 0.2). Among the 134 patients who survived, 43 (32%) remained hospitalized at the time of the convalescent-phase interview, 86 (64%) had returned home, and 5 (3.7%) were lost to follow-up; 5 patients had a seizure after discharge. Of the 86 persons who returned home, 52 (60%) reported complete cognitive recovery; of these, 45 (87%) of these functioned independently or at the same level of care as before hospitalization.

Discussion

In Thailand, a wide range of pathogens cause acute encephalitis. In this study, by using a comprehensive approach and advanced diagnostic methods, we identified a confirmed/probable etiology for only 36% of 149 patients, and >1 confirmed/probable pathogen was detected for 11% of these patients. Detection of possible pathogens was so common as to make interpretation challenging. Ten percent of patients died; highest mortality rate was among patients for whom etiology was classified as possible/unknown.

In our study, the most frequently identified pathogen (39% of all confirmed/probable etiologies) was JEV, which is endemic to Thailand; routine infant vaccination was introduced in 2001 (*28*). JEV data from our study have been published (*29*). Among patients for whom etiology was confirmed/probable, the etiologic pathogen was potentially treatable for 48%. Most of these 12 treatable pathogens, except herpes simplex virus, influenza viruses, *M. pneumoniae*, *O. tsutsugamushi*, and *R. conorii*, can be diagnosed in the study hospital laboratories in Thailand through routine CSF and blood culture, Venereal Disease Research Laboratory testing, or rapid antigen tests of CSF. The clinical features did not enable differentiation of specific etiologic agents. For many patients, a standard empiric treatment regimen consisting of a third- or fourth-generation cephalosporin, acyclovir, and doxycycline might be appropriate. The national reference laboratory in Thailand is able to test for all confirmed/probable etiologic agents.

This study highlights the clinical overlap between encephalitis and meningoencephalitis. Almost half of the patients met the definition for meningoencephalitis, yet for half of those patients, abnormalities detected by EEG or neuroimaging were consistent with encephalitis, and their illnesses were associated with a range of pathogens, some not typically associated with meningitis. Furthermore, we found that patients whose meningitis was caused by common bacteria, such as *S. pneumoniae* or *H. influenzae*,

Table 2. Clinical testing results for 149 patients meeting the case definition of encephalitis, Thailand, 2003–2005*

Hospital test	No. positive/ no. tested (%)	Organisms identified (no. if >1)
Blood		
Culture	21/129 (16)	*Acinetobacter* spp., *Escherichia coli,*[1] gram-negative bacilli,† gram-positive cocci (2),[2]† *Haemophilus influenzae*,[3]† *Klebsiella pneumoniae, Proteus mirabilis/Staphylococcus* coagulase negative, *Pseudomonas aeruginosa* (2),[4] *Salmonella* group D,[5]† *Staphylococcus aureus* (3),[6] *Staphylococcus* coagulase negative (5), *Streptococcus pneumoniae,*[7] *Streptococcus* not group A, B, D
Malaria smear	0/123	
VDRL	1/124 (1)	
HIV‡	8/137 (6)	
Toxoplasmosis antibody	0/2	
Cerebrospinal fluid		
Culture	19/141 (13)	*Bacillus* spp.,[6] *Cryptococcus, Cryptococcus/Acinetobacter* spp. (2), *E. coli*,[1] gram-negative nonfermenting bacilli† gram-positive cocci in clusters,† *Haemophilus influenzae* (2),[3]† *Neisseria meningitidis, P. aeruginosa*,[4] *Salmonella* group D,[5] *S. aureus*, *Staphylococcus*, coagulase negative (3), *S. pneumoniae* (2),[2,7] *Streptococcus viridans*
India ink stain	2/59 (3)	
Cryptococcal antigen test	1/31 (3)	
VDRL	0/6	
Latex agglutination		
Group B *Streptococcus*	1/43 (2)	
Neisseria meningitidis	0/54	
S. pneumoniae	3/55 (5)	
H. influenzae	3/55 (5)	
Escherichia coli	0/48	
PCR		
Herpes simplex virus	1/38 (3)	
Varicella-zoster virus	1/5 (20)	
Mycobacterium tuberculosis	4/30 (13)	
Sputum smear or culture		
M. tuberculosis	1/21 (5)	

*Superscript numbers indicate patients positive on blood and CSF culture for specified pathogens. GPA, gelatin particle agglutination; VDRL, Venereal Disease Research Laboratory. Blank cells indicate organism not applicable.
†Further identification not done.
‡6 were positive by ELISA (3 also by GPA, 2 also by GPA and immunochromatography, and 1 also by immunochromatography), 1 by GPA only, and 1 by an unrecorded assay.

sometimes met the case definition but did not meet the criteria for meningoencephalitis (pleocytosis and stiff neck). We reported laboratory results for all patients who met the encephalitis case definition, and we also repeated the analyses excluding the 8 patients with only a confirmed etiology of bacteria commonly associated with meningitis, which minimally affected the results. It could be argued that including pleocytosis in the case definition may have resulted in a predilection for enrolling patients with meningoencephalitis and/or meningitis; however, pleocytosis has commonly been included in the case definition for studies of encephalitis (*24*), and including it was necessary for our study because we were uncertain whether neuroimaging would be routinely performed.

Several pathogens that cause encephalitis were notably absent, including chikungunya, Nipah, and West Nile viruses; *B. henselae*; and malaria parasites. *B. henselae* infection occurs in Thailand, although it has not been widely studied (*30*). Because of extensive vector control efforts in Thailand, malaria parasite transmission is limited to areas along Thailand's borders with Burma and Cambodia, so it was not surprising that no cases of malaria were identified in Bangkok and Songkhla (*31*). Nipah virus has caused outbreaks of encephalitis in humans in Malaysia and Bangladesh; although it has been found in bats in Thailand, it has not yet been identified in humans (*32*,*33*). West Nile virus has also not yet been identified in Thailand.

Enteroviruses were found in CSF of 6 children 3 months to 10 years of age. Enteroviruses commonly cause aseptic meningitis and have also been clearly demonstrated to cause encephalitis. In Asia, epidemics of enterovirus 71 infection causing severe central nervous disease have occurred (*34*); however, enterovirus 71 was not detected in the CSF of any patient in our study. Consistent with reports in the literature, we found 2 cases of dengue virus infection (*35*). We found 6 cases of infection with *O. tsutsugamushi,* the etiologic agent of scrub typhus, which is prevalent in Southeast Asia although not thought to be common in Bangkok. Thus, the fact that 5 patients became ill while in central Thailand suggests that physicians should consider this pathogen in areajs outside the known disease-endemic southern provinces. Most cases of scrub typhus, and spotted fever caused by *R. conorii* (which we identified in 3 patients), can be treated effectively with doxycycline (*36*).

Table 3. Final classification of cases of encephalitis into etiologic category Thailand, 2003–2005*

Pathogen	Etiologic classification category			Total
	Confirmed	Probable	Possible	
Adenovirus	0	0	7†	7
Bartonella henselae	0	0	0	0
Chikungunya virus	0	0	0	0
Cryptococcus spp.	3	0	0	3
Dengue virus	2	0	0	2
Epstein-Barr virus	0	3	0	3
Enteroviruses	6‡	0	24§	30
Escherichia coli	1	0	0	1
Herpes simplex virus 1/2	1	0	0	1
Human herpesvirus 6	0	0	0	0
Human herpesvirus 7	0	0	1	1
Haemophilus influenzae¶	3	0	0	3
HIV	0	0	8	8
Influenza viruses	0	1	5	6
Japanese encephalitis virus#	15	6	0	21
Malaria	0	0	0	0
Measles virus	0	0	10	10
Mumps virus	0	0	8	8
Mycoplasma pneumoniae	0	3	1	4
Nipah virus	0	0	0	0
Neisseria meningitidis	1	0	0	1
Other bacteria	0	0	21**	21
Orientia tsutsugamushi (scrub typhus)	0	6	0	6
Parainfluenza viruses 1,2,3	0	0	5	5
Parasites, other	0	0	0	0
Rabies virus	0	0	1††	1
Rickettsia conorii (spotted fever)	0	3	0	3
Rubella	0	0	4	4
Salmonella group D	1	0	0	1
Streptococcus pneumoniae	3	0	0	3
Treponema pallidum	0	1‡‡	0	1
Varicella-zoster virus	2	0	0	2
West Nile virus	0	0	0	0
Total no. pathogens detected	38	23	95	156
Total no. patients	37	17	44	98

*Strategy used is same as that shown in Table 1. Pathogen categories are not mutually exclusive.
†Only 1 in a cerebrospinal fluid sample was positive by PCR and it was adenovirus type 40.
‡Echovirus 9 (n = 3); echovirus 27 (n = 2); echovirus 30 (1).
§Enterovirus 71 (n = 3); enterovirus, untyped (n = 21).
¶Not typed.
#This number is 1 less confirmed Japanese encephalitis case than previously published (*29*). The excluded patient had onset of neurologic symptoms 18 d after hospital admission.
**See Table 2 for organisms, excludes coagulase-negative *Staphylococcus*.
††Record says never vaccinated.
‡‡Serum Venereal Disease Research Laboratory testing only.

Unfortunately, most hospital laboratories do not routinely perform diagnostic serologic testing for these infections, so they might often not be suspected unless a prominent eschar or rash is visible. EBV was found in the CSF of 3 patients, concomitant with another pathogen for 2 patients. In these cases, EBV detection probably represented reactivation of latent EBV in the setting of a primary central nervous system infectious agent (*37*). Three cases of encephalitis were associated with *M. pneumoniae*, all in children 6–14 years of age, consistent with other study findings (*38*,*39*).

Comprehensive etiologic studies present many challenges and have limitations. In this study, CSF diagnostics were the most compelling approach for identifying an etiology; yet in some patients, CSF or other specimens were inadequate for complete testing for all agents. We used consensus primers to detect herpesviruses by PCR, but this method is probably less sensitive than one that uses specific primers for each herpesvirus. Feces and oropharyngeal swabs samples were not tested for enteroviruses, but these samples can be more likely to yield this pathogen. Last, we did not investigate noninfectious forms of encephalitis, such as anti–*N*-methyl-D-aspartate receptor and voltage-gated potassium channel antibody encephalitis, which have been shown in recent studies to cause encephalitis with poor outcomes (*7*,*40*).

Encephalitis is a severe disease that can cause substantial illness and death. Despite the high proportion of patients for whom large volumes of CSF and acute- and late convalescent–phase serum were tested and a broad range of routine and sophisticated diagnostic testing was performed, the etiology for one third of the patients remained unknown and for another one third was classified only as possible. The proportion of cases for which etiology

Table 4. Admission characteristics of 149 case-patients with encephalitis, according to etiologic classification, Thailand, 2003–2005*

Characteristic	Etiologic classification		p value
	Confirmed/probable, n = 54	Possible/unknown, n = 95	
Male sex, no. (%)	33 (61)	51 (54)	0.38
Age, y, median (range)	9 (0–74)	17 (0–82)	0.10
Time between onset of neurologic symptoms and admission, d, median (range)	1 (8–4)†	1 (12–7)†	0.35
Time between onset of fever and admission, d, median (range)	4 (31–1)†	4 (35–2)†	0.33
Temperature, °C, median (range)	38.0 (36.0–42.0)	37.7 (35.5–40.2)	0.03
Symptoms during illness, no. (%)			
Respiratory	12 (22)	27 (28)	0.41
Gastrointestinal	23 (43)	36 (38)	0.57
Rash	4 (7.4)	14 (15)	0.30
Glasgow Coma Scale score, no. (%)			0.44
≤8	7 (13)	18 (19)	
9–12	13 (24)	27 (28)	
≥13	34 (63)	50 (53)	
Mean (SD)	12 (3.2)	12 (3.7)	0.37
Neurologic signs/symptoms, no. (%)			
Alteration of consciousness	35 (65)	63 (66)	0.85
Lethargy	27 (50)	45 (47)	0.76
Seizure	19 (35)	48 (50)	0.07
Focal neurologic signs	19 (35)	32 (34)	0.89
Personality change	22 (41)	39 (41)	0.97
Somnolence	23 (43)	34 (36)	0.44
Loss of consciousness	8 (15)	28 (30)	0.04
Extreme irritability	9 (17)	23 (24)	0.28
Coma	10 (19)	15 (16)	0.67
Ataxia	2 (3.7)	6 (6.3)	0.71
Headache	29 (54)	45 (47)	0.46
Stiff neck	29 (54)	29 (31)	0.01
Pentobarbital or paralytic medications for intractable seizures	15 (28)	22 (23)	0.53
CSF pleocytosis, no. (%)	49 (91)	76 (80)	0.09
Abnormal MRI/CT/cranial U/S findings, no. (%)	23/31 (74)	57/69 (83)	0.33
Abnormal EEG, no. (%)	6/9 (67)	22/30 (73)	0.69
Length of hospital stay, median days (range)	13 (1–180)	16 (1–128)	0.71
Intensive care unit admission, no. (%)	15 (28)	33 (35)	0.38
Died, no. (%)‡	2 (3.7)	13 (14)	0.09

*CSF, cerebrospinal fluid; EEG, electroencephalogram, MRI, magnetic resonance imaging; CT, computed tomography; U/S, ultrasonography.
†Ranges indicate no. days before and after hospital admission.
‡All but 1 patient died while hospitalized.

was unknown in this study was slightly lower than that in other studies, perhaps because so many diagnostic tests were used (*1*,*10*–*12*,*26*). Because laboratory testing was performed simultaneously rather than sequentially, testing bias was reduced; however, because of multiple positive results, the complexity of interpretation was increased.

Although Thailand is a middle-income country with trained neurologists and more sophisticated medical and laboratory tools for diagnosing encephalitis than are found in many other countries in Southeast Asia, it still lacks the ability to routinely identify major causes of encephalitis. This study highlights the continuing role of Japanese encephalitis and other potentially preventable or treatable diseases in Thailand, such as those caused by the rickettsiae *O. tsutsugamushi* and *R. conorii*. Despite intensive efforts to diagnose cases, this study also emphasizes the need for improved diagnostic strategies and geographically appropriate clinical testing algorithms for adults and children with encephalitis in Southeast Asia. Such strategies and algorithms were recently presented by the International Encephalitis Consortium (*24*) to provide a standardized approach for the evaluation of patients suspected of having encephalitis. Such an approach will facilitate worldwide research collaboration and enable clinicians to provide appropriate clinical care for patients with this severe and often devastating neurologic syndrome.

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etymologia

Bonferroni [bon'fər-ō"ni] Correction

Named after Italian mathematician Carlo Emilio Bonferroni (1892–1960) but first attributed to Olive Jean Dunn, the Bonferroni correction compensates for multiple comparisons by dividing the significance level by the number of comparisons. The significance level is the probability that a given test will incorrectly find a difference in the sample that is not present in the population (false positive). A significance level of 0.05 is a commonly accepted significance level. If a study tested 5 comparisons, there would be up to a 25% likelihood (0.05 + 0.05 + 0.05 + 0.05 + 0.05) that any one of them would show a significant difference by chance. The Bonferroni correction adjusts for this by dividing the significance level by the number of tests. In this case, the significance level for a given comparison would be 0.01, for an overall risk no larger than 0.05 of falsely detecting a difference.

This technique has been criticized as too conservative, particularly when a large number of tests are used, and it may increase the risk for a false negative. Other tests, such as the Tukey-Kramer and Scheffe method, may reduce this risk.

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Lagenidium giganteum Pathogenicity in Mammals

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Infections of mammals by species in the phylum Oomycota taxonomically and molecularly similar to known *Lagenidium giganteum* strains have increased. During 2013–2014, we conducted a phylogenetic study of 21 mammalian *Lagenidium* isolates; we found that 11 cannot be differentiated from *L. giganteum* strains that the US Environmental Protection Agency approved for biological control of mosquitoes; these strains were later unregistered and are no longer available. *L. giganteum* strains pathogenic to mammals formed a strongly supported clade with the biological control isolates, and both types experimentally infected mosquito larvae. However, the strains from mammals grew well at 25°C and 37°C, whereas the biological control strains developed normally at 25°C but poorly at higher temperatures. The emergence of heat-tolerant strains of *L. giganteum* pathogenic to lower animals and humans is of environmental and public health concern.

During the 20th century, *Pythium insidiosum* was the only fungus-like species from the phylum Oomycota known to cause life-threatening infections in mammals and birds (*1*,*2*). In 2003, a novel group of pathogenic Oomycota was isolated in the southeastern United States from dogs with invasive cutaneous infections resembling pythiosis, but the strains had morphologic and molecular affinities with the genus *Lagenidium* (*3*,*4*). Subsequently, other strains similar to previously studied *Lagenidium* species were found to be associated with infections in dogs, cats, and humans (*5*–*7*). The disease caused by these 2 Oomycota species has been reported mainly in apparently healthy hosts; thus, these species' evolutionary traits need to be investigated (*1*–*7*). Although detailed descriptions of the clinical, pathologic, and serologic features of *Lagenidium* spp. pathogenic to mammals have been published (*3*,*4*), their taxonomic relationship to *Lagenidium* strains approved for biological control and the capacity of their zoospores to infect mosquitoes have yet to be investigated (*5*,*6*).

The genus *Lagenidium*, introduced by Schenk in 1857 (*8*), comprises numerous saprotrophic species (*9*), as well as several members that are pathogenic to algae, phytoplankton, pollen, barnacles, blue crabs, mosquito larvae, shrimp, and mammals (*3*–*5*). Couch (*10*) isolated *L. giganteum* from *Daphnia* larvae in Virginia, USA. Later, Couch and Romney (*11*) confirmed that *L. giganteum* infects and kills mosquito larvae as it reproduces sexually. Their work stimulated interest in *L. giganteum* as a biological control agent of mosquitoes (*11*,*12*). In 1995, the US Environmental Protection Agency registered *L. giganteum* under the trade name Laginex as a biocontrol agent (*13*) but later deregistered it at the request of the manufacturer (http://www.gpo.gov/fdsys/pkg/FR-2011-09-28/pdf/2011-24832.pdf). *L. giganteum* strains used for testing and commercial development were deposited within the American Type Culture Collection (ATCC), and the ATCC strains and other not-well-characterized strains were released in nature for field studies (*12*–*16*). In this study, conducted during 2013–2014, we investigated 21 mammalian *Lagenidium* strains and compared them with *L. giganteum* isolates used for mosquito control.

Materials and Methods

Living Cultures

A complete list of the strains and their accession numbers is shown in the Table (http://wwwnc.cdc.gov/EID/article/21/2/14-1091-T1.htm). In brief, we studied the following isolates: *L. giganteum* (ATCC 36492 = ATCC 52675 used as biological control and ATCC 48336 isolated from mosquito larvae); *L. humanum* (ATCC 76726 isolated from the skin of dead humans); *L. giganteum* pathogenic to mammals (culture collection at the Biomedical Laboratory Diagnostics, Michigan State University (MTLA): MTLA 01 = ATCCMYA-4933 type strain, MTLA-03, MTLA-04 = ATCCMYA-4934 (from a US cat), MTLA-05 = ATCCMYA-4935, MTLA-10, MTLA-12, MTLA-14, MTLA-15, MTLA-16, MTLA-17, and MTLA-18 from dogs); *L. ajelloi* (MTLA-06 = ATCCMYA-4936 type strain, MTLA-07 = ATCCMYA-4937, MTLA-19, MTLA-20, MTLA-21, MTLA-22, MTLA-23, isolated from dogs); *L. vilelae* (MTLA-24 = DNA extracted from fixed feline tissue and MTLA-25 cat strain); and *L. albertoi* (MTLA13 = ATCCMYA-4932 type strain).

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Media and Culture Conditions

The isolates were grown on brain-heart infusion (BHI) (DIFCO, Detroit, MI, USA), corn meal agar (CMA) (BBL, Sparks, MD, USA), 2% Sabouraud dextrose agar (SDA), 2% Sabouraud dextrose broth, in triplicate. Cardinal temperatures of growth (25°C, 30°C, and 37°C measured during 3 days' incubation) and their relation to the production of sexual and asexual structures were evaluated on the above media. Development of the different stages of vesicle and zoospore formation, cleavage, and release were assessed on colonized grass leaves in water cultures containing Ca^{++} and Mg^{++}. Briefly, the evaluated strains (Table) were subcultured on SDA plates at 37°C for 24 h. After incubation, 5 × 5–mm diameter blocks were cut from the advancing edges of the culture and placed on top of a 2% water agar plates. Sterile 4 × 10–mm grass blades were laid on top of each block and incubated at 37°C for 24 h (*L. giganteum* = MTLA-01 from mammals). The grass blades were then collected and placed in a beaker that contained 50 mL of sporulation solution (made of 2 solutions: mix no. 1 contained $NH_{42}HPO_4$ [66.04 g], KH_2PO_4 [68.05 g], and K_2HPO_4 [87.09 g] in 500 mL H_2O; and mix no. 2 contained $CaCl_2.2H_2O$ [18.38 g] and $MgCl_2.6H_2O$ [25.42 g] in 250 mL H_2O). The sporulation solution was obtained by mixing 0.5 mL of solution no. 1 plus 0.1 mL of solution no. 2 in 1.0 L of distilled water. The beaker with the 50-mL of sporulation solution plus the parasitized grass blades was incubated at 37°C, and the development of sporangia and zoospores microscopically was evaluated every 30 min for the following 6 h (or longer when needed).

Morphologic Description of *L. giganteum* from Mammals

We evaluated the morphologic features of *L. giganteum* strains after subculture on BHI, CMA, and SDA at different intervals during 5 days' incubation at 37°C or at room temperature (25°C). Briefly, 4 × 4–mm agar blocks were cut from the above media and mixed with 1 drop of lactophenol cotton blue (phenol 20 mL, lactic acid 20 mL, glycerol 40 mL, and distilled water 20 mL). The morphologic features of their hyphal and other structures developed in these media, including oogonia, were microscopically investigated. We evaluated the development of sporangia and zoospores in sporulation medium (see above). After incubation at the induction temperatures, the beaker containing 50 mL of sporulation medium plus the parasitized grass blades was inspected on an inverted microscope. If vesicles and zoospores developed, we removed the grass blade with ≥10 vesicles, using tweezers, and placed it on a glass slide. Immediately, 5 μL of Merthiolate (0.02%) was added to the grass blade to stop the movement of the zoospores and facilitate measurements. Alternatively, grass blades containing vesicles with zoospores were also transferred to plates holding *Culex pipiens* larvae.

Experimental Mosquito Infection

The strains of *L. giganteum* from mammals (MTLA01, MTLA03, MTLA04, MTLA05, and MTLA10) (Table) were used to evaluate their capability to infect mosquito larvae of *C. pipiens*. Instar 3 *C. pipiens* larvae were collected and placed in 6-well plates (1 plate per experiment; Corning Inc., Corning, NY, USA) containing 5 mL of water and fed every other day with fish food flakes (TetraMin, Lancaster, PA, USA) until the end of the experiment. We transferred fresh grass blades, with ≥10 vesicles per blade containing numerous zoospores, to the plates holding 5 mosquito larvae per well (total of 30 larvae per plate). The plates were incubated at 25°C and observed daily by using an inverted microscope for the next 3 weeks. The criteria to select putative infected mosquito larvae were based on their swimming capabilities. We closely inspected slower swimmers for filamentous structures outside and between the larval segments and inside their bodies. Every trial included its positive (*L. giganteum* biological control ATCC 36492 = ATCC 52675) and negative controls (larvae without exposure to *L. giganteum* species), and the experiment was repeated twice (total 60 *C. pipens* larvae).

DNA Extraction and Molecular Procedures

The 20 strains investigated (Table) were inoculated into 250-mL flasks containing 100 mL of Sabouraud dextrose broth and incubated for 72 h at 37°C on a shaker rotating at 150 rpm. In addition, 1 formalin-fixed tissue sample from a cat was also used to extract total genomic DNA following the company protocols (QIAGEN, Valencia, CA, USA). After incubation, the cultures were killed with Merthiolate (0.02%, wt/vol) and then filtrated to separate the hyphal cell mass. The hyphal cell mass was then transferred to a mortar and ground in the presence of liquid nitrogen. DNA from the disrupted hyphae was treated with sodium dodecyl sulfate and proteinase K and then incubated at 60°C for 1 h, and genomic DNA of the hydrae was extracted with phenol, chloroform, isoamyl alcohol (Sigma, St. Louis, MO. USA). PCR was conducted by hot start amplification using the following primers: the universal primers for internal transcribed spacers (ITS): ITS1 = TCCGTAGGTGAACCTGCGG and ITS4 = TCCTCCGCTTATTGATATGC; cytochrome oxidase II (COXII): COX.LagF = 5′-CCACAAATTTCACTACATTGA-3′ and COX.LagR = 5′-TAGGATTTCAAGATCCTGC-3′; heat shock protein 90 (HSP90): HSP90.LagF = 5′-CAACCTBGGHACSATYGCCAAG-3′ and HSP90.LagR = 5′-ACRAAMGACARGTAYTCVGGCA-3′; cell division cycle 42 (CDC42): CDC42.LagF = 5′-GTSCCVACYGTVTTYGANAAYTA-3′ and CDC42.LagR = 5′-GCWSWGCAYTCVASRTAYTT-3′; Tubulin: TUB.LagF = 5′-GGTGGTGGTACCGGTTC-3′ and TUB.LagR

= 5′-GACACACGCTTGAACATC-3′. In addition, the following primers were constructed to PCR amplify some of the *L. ajelloi* and *L. vilelae* strains: CDC42.Lag2F = 5′-GTGCCGACYGTGTTYGABAAC-3′ and CDC42. Lag2R = 5′-CTSTTCKGTTGTRATBGG-3′; and HSP90. Lag2F = 5′-GAGGCCTTCGTGGAAGCG-3′ and HSP90. Lag2R = 5′-GTTGTTCATTTTCTTGCGGG-3′. The PCR temperature-cycling parameters were as follows: 10 min at 95°C and 1 min for subsequent cycles, annealing for 1 min at 60°C, and elongation at 72°C for 2 min. The parameter was repeated for 40 cycles, followed by a final elongation of 2 min at 72°C for 7 min. The amplicons were ligated into pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA. USA), purified, and then sequenced by using BigDye Terminator chemistry in an ABI Prim 310 genetic analyzer (Perkin-Elmer, Foster City, CA. USA).

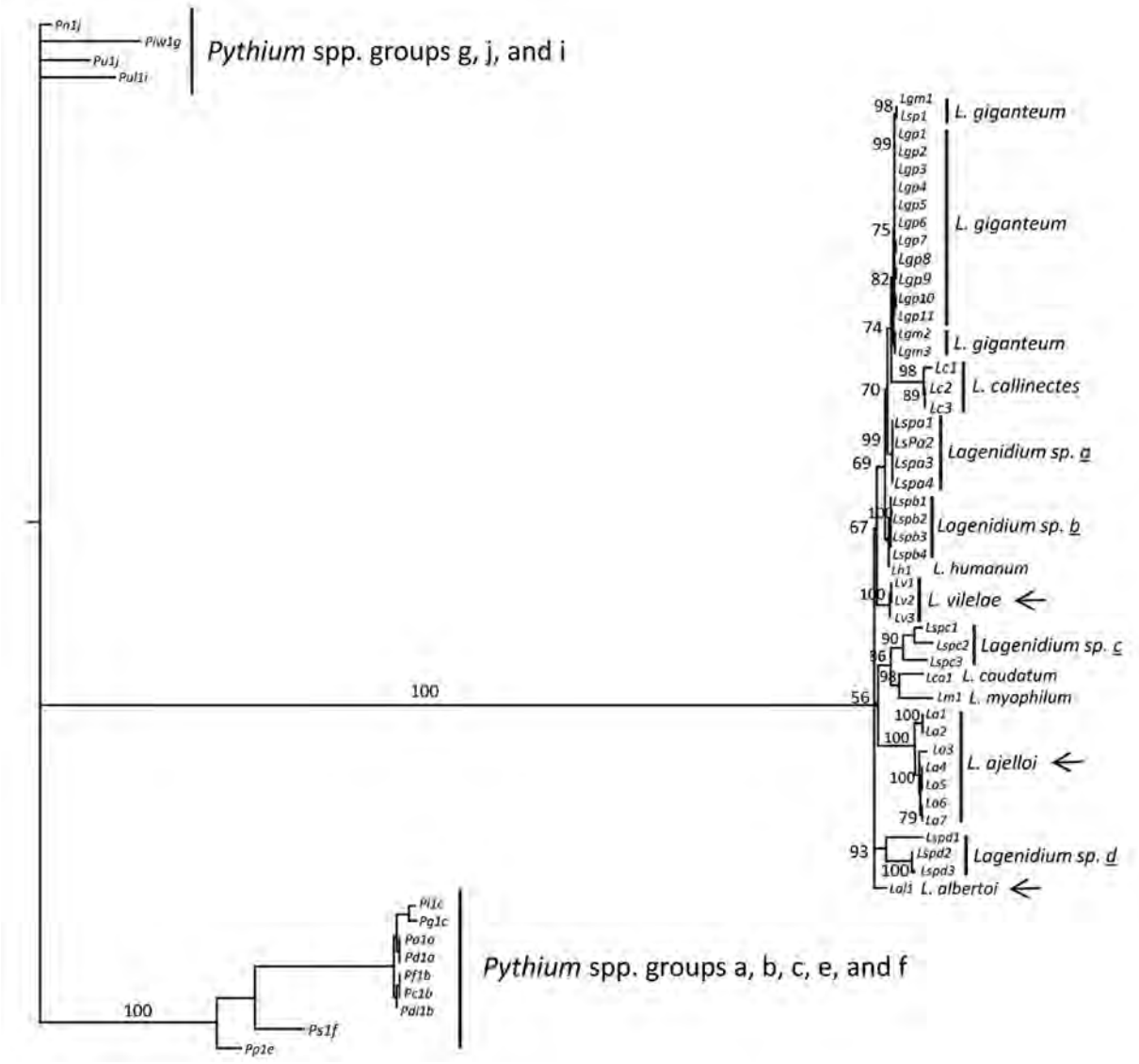

Figure 1. Bayesian phylogenetic analysis of concatenated 4 partial coding gene sequences (cell division cycle 42, cytochrome oxidase II, heat shock protein 90, and tubulin) and the complete internal transcribed spacers 1, 2, and 5.8S of *Lagenidium* DNA sequences. Thirteen *Pythium* species DNA sequences were included as the outgroup (groups a–c, e–g, j, and l [*16*]; Table, http://wwwnc.cdc.gov/EID/article/21/2/14-1091-T1.htm). Support on key branches is the Bayesian probability for that branch followed by the percentage of 1,000 bootstrap resampled datasets containing the branch in neighbor-joining analyses of maximum-likelihood distances followed by the percentage of 1,000 bootstrap resampled datasets containing the branch in parsimony analyses using heuristic searches. In this analysis, the DNA sequences of *L. giganteum* mosquito control (Lg 1–3) and a *Lagenidium* sp. recovered from a nematode in Taiwan (Ls1, Lsp1 = HQ395647) clustered with *L. giganteum* from mammals (Lg 1–10). The pathogen of crab *L. callinectes* (Lc) was the sister group to the cluster. Three *Lagenidium* mammalian pathogenic novel species (*L. ajelloi* = La, *L. albertoi* = Lal, and *L. vilelae* = Lv) were placed in 3 distinctive strongly supported clades (arrows). The accession numbers, the abbreviations used to identify each species, and the *Lagenidium* and *Pythium* spp. DNA sequences are shown in the Table. ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures.

Phylogenetic Analysis

Genomic DNA sequences of the studied strains' complete ITS and the partial gene sequences of the selected 4 exons (*CDC42*, *COXII*, *HSP90*, and *TUB*) were aligned with other *Lagenidium* and *Pythium* DNA sequences available at the National Center for Biotechnology Information by using ClustalW version 1.81 (http://www.clustal.org)with default settings and their alignments visually inspected. *Pythium* spp. groups a–c, e–g, and j (*15*) (Table) were used as outgroups. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA6 (http://www.megasoftware.net). The aligned sequences were exported for parsimony analysis by using a heuristic search with tree bisection reconnection branch swapping (MEGA6) and distant analysis by neighbor-joining (MEGA6). We coded large insertions as 1 event by excluding all but 1 nt per insertion. The generated gaps were treated as missing data. Neighbor-joining analysis used either uncorrected distances or maximum-likelihood estimates of distance with a time-reversible model (6ST), and empirical base frequencies with no rate variation among sites, or a shape parameter of 0.5 γ-distribution with 4 rate categories. Branch support was estimated as percentage of parsimony tree (1,000 resampling, heuristic, branch swapping) or neighbor-joining trees (1,000 resampling, maximum-likelihood distances) within each branch. Concatenated DNA sequences of the 5 loci investigated were used in Bayesian analysis. We conducted the test in MrBayes version 3.2.1 ×64 (http://mrbayes.sourceforge.net) using the general time reversible + I + γ model, with 2 chains (1 heated), 2 runs, sampling

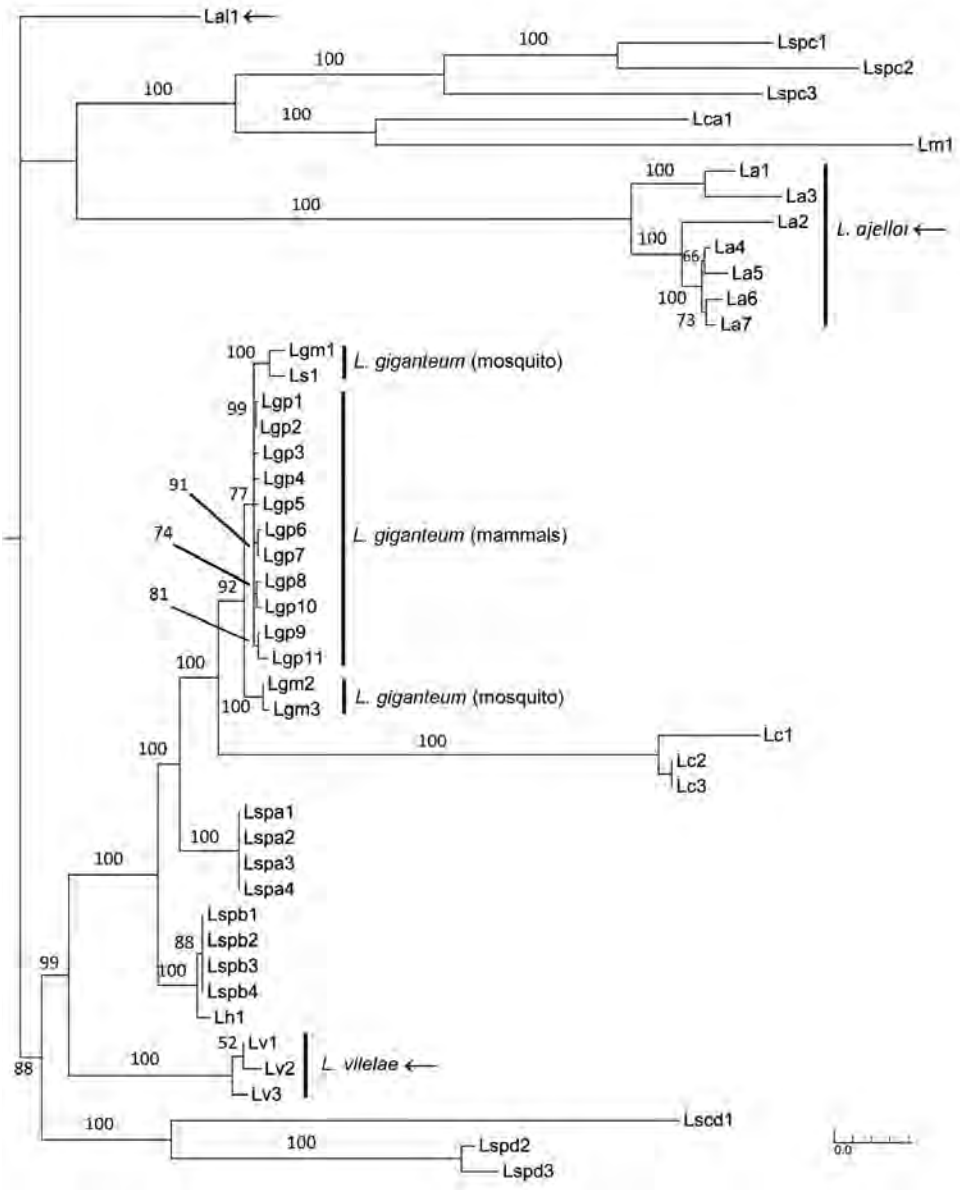

Figure 2. The Bayesian tree was constructed by concatenated aligned of *Lagenidium* spp. DNA sequences as in Figure 1 without outgroup to highlight the position of *L. giganteum* in the tree. *L. giganteum* from mammals (Lgp 1–11), *L. giganteum* mosquito control (Lgm 1–3 and Ls1 = HQ395647), and the novel species *L. ajelloi* = La, *L. albertoi* = Lal, and *L. vilelae* = Lv were placed in 4 strongly supported clades (arrows). Scale bar indicates nucleotide substitutions per site.

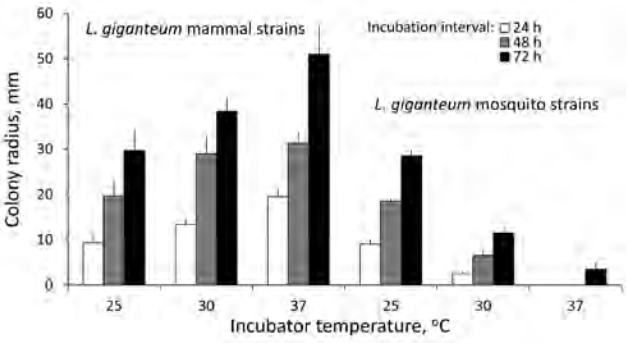

Figure 3. Cardinal temperatures of *Lagenidium giganteum* types in culture. Growth (mean colony radius and SEM, mm) of *L. giganteum* mammalian and mosquito strains at 3 temperatures at 24-, 48-, and 72-hour intervals postinoculation onto 2% Sabouraud dextrose agar. Repeated measures analysis of variance showed highly significant differences between strains ($F_{1,33} = 165.0$, $p<0.0001$) and a highly significant interaction of strain and incubation temperature across time intervals ($F_{2,33} = 45.9$, $p<0.0001$).

every 100th generation for 1×10^6 generations, and exclusion of the first 2.5×105 samples (the burn-in) before analysis. Support for branches was estimated as the percentage of parsimony tree (1,000 resampling, heuristic, nni branch swapping) or neighbor-joining tree (1,000 resampling, maximum-likelihood distances) containing the branch, as well as by determining the Bayesian probability estimated as the percentage of Bayesian trees possessing the branch after discarding the burn-in sample.

Results

Phylogenetic Analysis

Using *Pythium* spp. groups a–c, e–g, and j (Table) as outgroups, we sorted the concatenated DNA sequences of 4 protein-coding genes and 1 ribosomal region in Bayesian and maximum-likelihood phylogenetic analyses 21 mammalian pathogenic *Legenidium* strains into 4 strongly supported clades in the combined analysis (Figure 1). In the largest of these clades, 11 of the 21 strains (Table) recovered from infected mammals form a monophyletic clade that includes other *L. giganteum* strains previously used to develop biological control agents of mosquitoes (Figure 1). Also included in this clade is an *L. giganteum* strain recovered from nematodes in Taiwan (Lsp1). The remaining 10 mammal-infecting *Lagenidium* strains were resolved with strong support into 3 independent clades that are basal to *L. giganteum* and identified in this study as *L. ajelloi*, *L. albertoi*, and *L. vilelae* (Figures 1, 2). These new species will be fully described elsewhere. Other clades of *Lagenidium* species, as yet unnamed, are also apparent in the phylogeny (Lspa to Ld; Figures 1, 2).

Heat-tolerance and Mosquito Larva Infection Experiments

The 11 *L. giganteum* strains from mammalian hosts grew faster at 37°C and 30°C than at 25°C, whereas *L. giganteum* biological control strains (ATCC 36492 = ATCC 52675 and ATCC 48336) grew well at 25°C and poorly at 30°C and almost failed to develop at 37°C in growth experiments conducted in CMA and SDA media (Figure 3). The biological control strains barely developed at 37°C but survived, as shown by their subsequent growth, after being transferred to 25°C.

We tested the ability of the mammalian *L. giganteum* zoospores to infect mosquito larvae in 5 *L. giganteum* mammal strains (MTLA01, 03, 04, 05, and 10; Table) and 2 *L. giganteum* biocontrol strains (ATCC 48336 = Lgm2, ATCC 36492 = Lgm3; Table). The emerging mammalian pathogenic strains exhibited mosquito-infection capabilities similar to those of the strains approved by the US Environmental Protection Agency for mosquito control (Figure 4). At least 1 larvae per well was found infected. Infected larvae exhibited abnormal swimming behavior and died within 2 days (Figure 4). Of the 60 *C. pipiens* larvae (2 trials, 30 larvae per experiment), 12 were found infected at the end of both experiments (instar 3 or instar 4). Uninfected larvae continued their normal life cycle. Postmortem examination of infected larvae showed extensive infections with numerous hyphae spreading internally and emerging between segments (Figure 4, panels A–E). Under our experimental conditions, *L. giganteum* ATCC 36492 infected 18 of the 60 *C. pipens* larvae. In unexposed controls all larvae survived, larvae swam normally, and we found no evidence of infection. The identity of mammalian *L. giganteum* strains experimentally infecting mosquito larvae was confirmed by culture, DNA extraction, sequencing, and phylogenetic analysis at the conclusion of each experiment.

Morphologic Characteristics of *L. giganteum* in Culture

Submerged colonies of both *L. giganteum* types (biological control and mammalian strains) are colorless or yellow with few aerial mycelia that present irregular radiating and undulating patterns on any of 3 media (CMA, BHI, or 2% SDA) (Figure 5, panels A, D). Typically, the elongated mycelium comprises ovoides or spherical 30- to 40-μm diameter segments forming chains and connected by broad to slender isthmuses; hyphae also form long irregular segments of ≥400 μm (Figure 5, panels B, C, E, F). We did not find oogonia in the evaluated strains. In liquid sporulation medium, hyphal segments develop 1–2 discharge tubes that produce terminal vesicles containing reniform zoospores 9–10 μm wide × 10–12 μm long (Figure 5, panel G). Zoospores were released in both mammal and mosquito *L. giganteum* strains after the discharge vesicle was disrupted (Figure 5, panel H), which then swam for 10–20 minutes

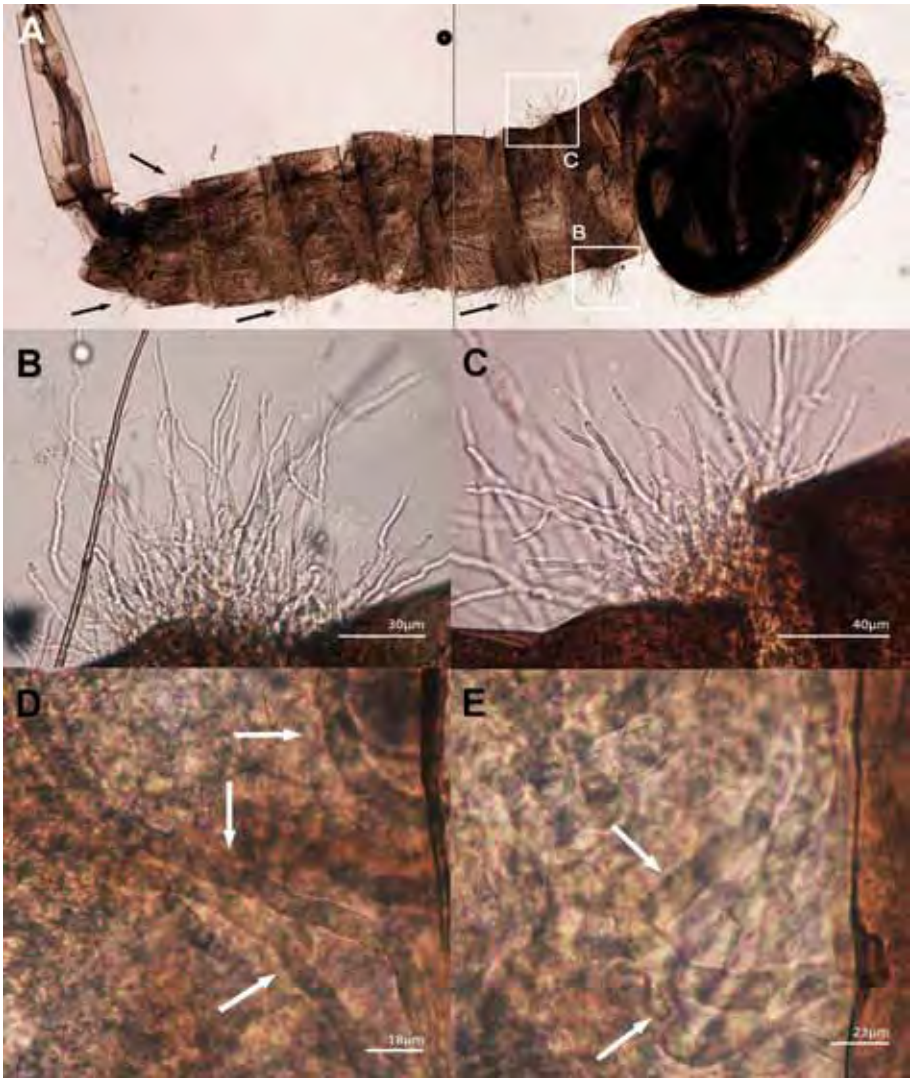

Figure 4. *Lagenidium giganteum* from mammal experimental infection using *Culex pipiens* mosquito larvae. A) Composite of 2 photographs showing an instar 3 *C. pipiens* larvae infected with 1 of the 5 tested strains of *L. giganteum* recovered from dogs with lagenidiosis (MTLA01, type strain). Note the mycelioid structures emerging from the infected larvae (arrows). B, C) Enlargements of the 2 white boxes in (A) showing details of the mycelioid structures emerging between the segments of the larvae. D, E) Aggressiveness of the invading mycelioid structures within the body of *C. pipiens* larvae.
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before encysting. Germ tubes developed from encysted zoospores within 10–20 minutes after encystment.

Discussion

We detected a novel group of mammalian pathogenic *L. giganteum* strains unique for their capacity to experimentally infect invertebrate hosts and cause life-threatening infections in healthy lower animals and humans (*5*,*7*). Placement of both heat-sensitive (affecting mosquito larvae) and heat-tolerant (affecting mammals) *L. giganteum* strains in a strongly supported monophyletic clade in Bayesian analysis corroborates the taxonomic and phenotypic attributes shared by both types in this and other studies (*3–5*,*10–15*). In addition to *L. giganteum*, 3 other clades harbor mammal isolates: *L. ajelloi*, *L. albertoi*, and *L. vilelae* (Figures 1, 2). Strains in the remaining clades are from invertebrates, whether from nematodes (the 4 unnamed species, *L. spa*, *L. spb*, *L. spc*, and *L. spd* [Table]), crab eggs (*L. callinectes*), or shrimp (*L. myophilum*) (*17*). *Lagenidium* strains in the 3 clades closest to *L. giganteum* infect invertebrates, as can all isolates of *L. giganteum*, suggesting that the mammal-infecting *L. giganteum* isolates recently evolved the ability to infect warm-blooded hosts. However, the capacity to infect mammals is not unique to *L. giganteum*, and knowing that each of the 3 other mammal-infecting *Lagenidium* clades has closely related relatives that infect invertebrates, it seems likely that the trait of infecting mammals has arisen several times, independently, in the genus *Lagenidium*. From its name, 1 species, *L. humanum*, might be expected to be among the mammal parasites, but it has been isolated only from the dead skin of humans or snakes and cannot grow at 37°C (*9*). Regarding the trait of heat tolerance, species that are isolated from mammals, *L. ajelloi*, *L. albertoi*, *L. giganteum*, and *L. vilelae* also can grow at 37°C, but judging from cultures recovered from lower animals,

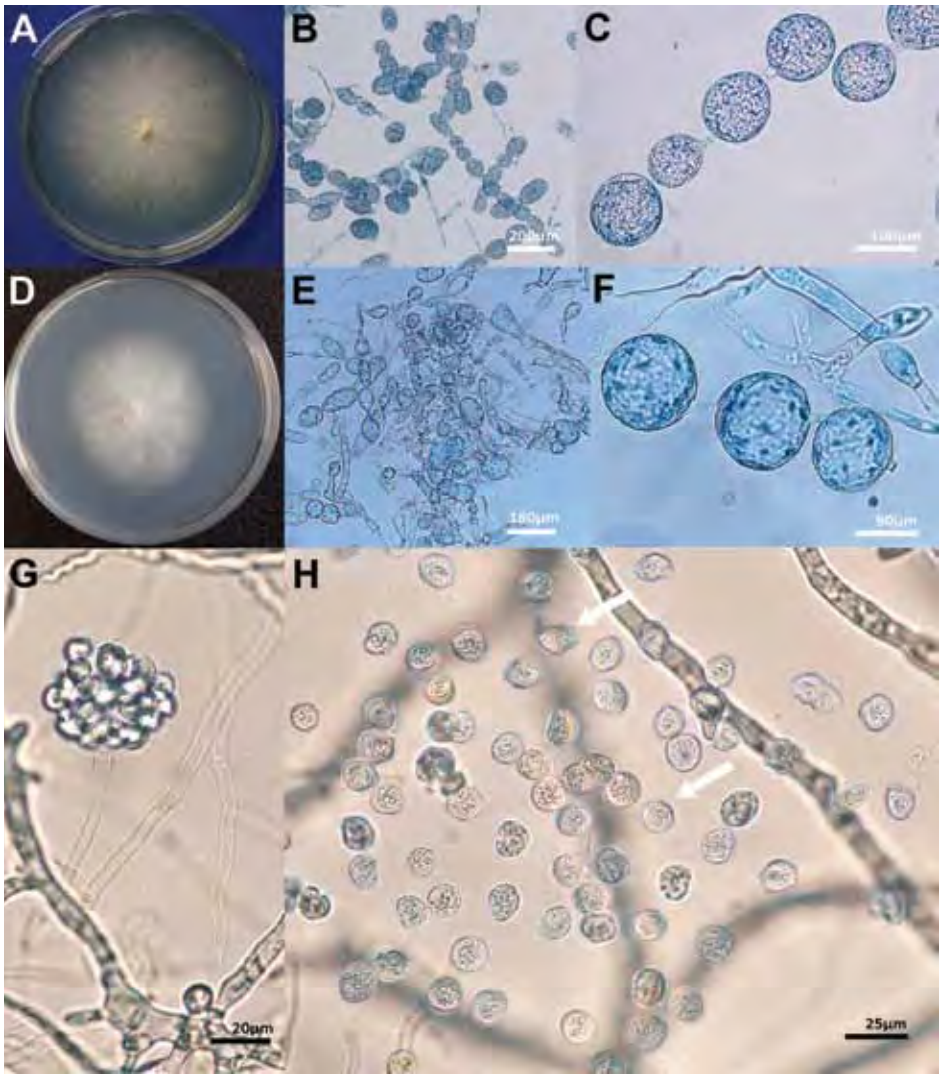

Figure 5. Morphologic features of isolates of *Lagenidium giganteum* mosquito control agent and *L. giganteum* mold from mammals. Panel A shows phenotypic features in culture of the mammalian pathogen (ATCCMYA-4933, type strain), and panel D shows the biological control (ATCC 36492). The development of spherical and ovoid 40- to 170-µm swelling segments (panels B, C, E, F) was the main feature of both mammalian and biocontrol strains. Panel G shows a tubular body developed from an unseen segmented sporangium form a vesicle enclosing numerous zoospores in a mammalian *L. giganteum* strain. The kidney-shaped zoospores before release (G) and after release (H, arrows) agree with those in the original description of *L. giganteum* by Couch (*10*). ATCC, American Type Culture Collection.

some strains associated with invertebrates also might lack the heat-tolerance trait. *Lagenidium* strain Centraalbureau voor Schimmelcultures (CBS) 127042 recovered from nematodes and phylogenetically linked to *L. vilelae* may be a good example of this unusual feature (Figure 1).

Emergence of mammalian pathogens often is accompanied by host switching in a zoonotic context from 1 vertebrate species to another (*18*,*19*), but range extension from invertebrates to vertebrates as hypothesized here is rare. Waterfield et al. (*20*) outlined 3 scenarios in which bacteria pathogenic to humans might have evolved from pathogens associated with invertebrates, but the time for the process was thousands of years, whereas in our second scenario, we describe a process that might have happened within a decade. The 2 phenotypes of heat-tolerance and mammal pathogenicity appear to have repeatedly evolved in the genus *Lagenidium*, and several recent studies have shown that members of Oomycota can acquire pathogenicity genes by horizontal gene transfer (*21*–*23*). Before their release as agents of biocontrol, *L. giganteum* (ATCC 52675 = ATCC 36492) (*12*) and other strains proposed as biocontrol agents of insect were shown not to infect other arthropods and mammals (*14*,*15*). However, experimental infection of mammals by the other Oomycota pathogen, *P. insidosum*, is difficult to achieve, e.g., only rabbits have been successfully, experimentally infected by this pathogen of cats, cattle, dogs, horses, and humans (*2*). This experience indicates that the safety of *Lagenidium* species as pathogens of hosts other than mosquito might be difficult to assess because of a general difficulty over experimental infection of mammals by Oomycota species.

The evolutionary relationships among *L. giganteum* strains should be more thoroughly examined by using population genomics, which could lead to discovery of the basis of adaptation to specific hosts, as has been shown for other adaptive phenotypes in other filamentous microbial eukaryotes (*24*).

The resent transcriptome analysis of a strain of *L. giganteum* recovered in nature from mosquito larvae further strengthens the relevance of our findings (*25*). The use of varieties or subspecies to name these 2 populations of *L. giganteum* was avoided until more comprehensive genomic data shed light on their true evolutionary relationships. Future genomic studies also could discriminate between the scenarios of gaining novel traits in specific ecologic niches or revealing types with hidden capabilities, both of which are of environmental and public health concern when the traits include putative pathogenicity of humans.

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Novel Reassortant Influenza A(H5N8) Viruses among Inoculated Domestic and Wild Ducks, South Korea, 2014

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An outbreak of highly pathogenic avian influenza, caused by a novel reassortant influenza A (H5N8) virus, occurred among poultry and wild birds in South Korea in 2014. The aim of this study was to evaluate the pathogenesis in and mode of transmission of this virus among domestic and wild ducks. Three of the viruses had similar pathogenicity among infected domestic ducks: the H5N8 viruses were moderately pathogenic (0%–20% mortality rate); in wild mallard ducks, the H5N8 and H5N1 viruses did not cause severe illness or death; viral replication and shedding were greater in H5N8-infected mallards than in H5N1-infected mallards. Identification of H5N8 viruses in birds exposed to infected domestic ducks and mallards indicated that the viruses could spread by contact. We propose active surveillance to support prevention of the spread of this virus among wild birds and poultry, especially domestic ducks.

Wild birds in orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, shore birds) are the natural reservoirs of avian influenza viruses (*1*,*2*). In wild aquatic birds, low pathogenicity avian influenza viruses are in a state of evolutionary equilibrium, and infected hosts usually show no signs of disease. However, a Qinghai-like H5N1 virus caused an outbreak in migratory waterfowl during 2005 before spreading from Asia to Europe and Africa (*3*,*4*). The outbreak gave rise to concerns that infections of wild birds with the highly pathogenic avian influenza (HPAI) virus subtype H5N1, which causes mild or no clinical signs in these birds, could result in transmission of the virus over long distances (*5*,*6*).

As was the case in other wild birds, HPAI H5N1viruses were not known to be pathogenic in domestic ducks before 2002 (*7*–*9*), but since then, HPAI H5N1 viruses that are pathogenic in ducks have been isolated in many countries (*3*,*5*,*10*,*11*). In South Korea, 4 outbreaks of HPAI H5N1 have occurred among poultry (mainly chickens and domestic ducks) and wild birds. Before 2010, H5N1 HPAI viruses among birds were detected mostly in poultry (chickens, domestic ducks, and quail), with the single exception of 1 magpie in 2004. By contrast, during 2010–2011, many cases occurred in various wild birds such as the Eurasian eagle owl, mandarin duck, Baikal teal duck, mallard duck, whooper swan, spot-billed duck, sparrow hawk, common kestrel, and white-fronted goose, as well as in poultry. Although all viruses in these outbreaks were highly pathogenic in chickens, the pathogenicity of these viruses varied among domestic ducks; the pathogenicity was 0%–25% during 2003–2004 (clade 2.5, H5N1), 0% during 2006–2007 (clade 2.2, H5N1), 50%–100% during 2008 (clade 2.3.2.1, H5N1), and 50%–100% during 2010–2011 (clade 2.3.2.1, H5N1) (*5*,*12*–*15*).

Outbreaks of HPAI H5N8 infection in poultry were first reported in ducks and turkeys in Ireland in 1983. In 2010, outbreaks of infection with the HPAI H5N8 virus derived from the Goose/Guangdong/1/1996 (Gs/GD) lineage were first reported in duck farms in Jiangsu, China, (*16*). Early in 2014, an outbreak of HPAI caused by a novel reassortant H5N8 virus occurred in Korea. The virus belonged to clade 2.3.4.6 and comprised 2 distinct genotypes (*17*). It has been suggested that viruses belonging to the major genotypes Buan2 and Donglim3 might be reassortants containing the polymerase basic protein 2, hemagglutinin (HA), nucleoprotein, and neuraminidase (NA) genes from viruses in the outbreak in China during 2010 (A/duck/Jiangsu/k1203/2013 (H5N8) (*17*). The HPAI H5N8 viruses were isolated from both wild birds and poultry. They were found in captured, apparently healthy, migratory wild birds and in dead birds, including mallards (both alive and dead), and in domestic chickens, geese, and ducks; the outbreak positive rate on duck farms was >70% (*13*).

There have been no previous reports about the pathogenicity of novel reassortant H5N8 isolates in wild birds and domestic ducks. Therefore, the aim of this study was to evaluate the pathogenesis in and mode of transmission of a novel reassortant H5N8 virus among mallard and domestic

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ducks, which are the poultry populations primarily affected by these viruses in South Korea.

Materials and Methods

Animals

Two species of wintering migratory birds, i.e., mallard ducks (*Anas platyrhynchos*) and Baikal teal ducks (*Anas formosa*), and 1 commercially available domestic bird (2-week-old Pekin ducks) were used in this study. The mallards and Baikal teals were captured in the wild and acquired through Konkuk University (Seoul, South Korea) and the Veterinary Epidemiology Division of the Animal and Plant Quarantine Agency. Both male and female ducks of each species were included and were approximately equally represented. All wild birds and domestic ducks used in this study were subjected to the H5 hemagglutination-inhibition (HI) test during a period of 1 week before experimentation and were maintained according to the guidelines of the Institutional Animal Care and Use Committee of Korea. All experiments were performed in a biosafety level 3–enhanced facility at the Animal and Plant Quarantine Agency, South Korea.

Viruses

The pathogenicity of the H5N8 virus was evaluated in mallards, Baikal teals, and domestic ducks. The virus strain A/breeder duck/Kr/Gochang1/2014 (H5N8) [Gochang1] was isolated from a breeder duck in which the index case was diagnosed during the 2014 South Korean outbreak. A/broiler duck/Kr/Buan2/2014 (H5N8) [Buan2] and A/Baikal teal/Kr/Donglim3/2014 (H5N8) [Donglim3], the main strains circulating in South Korea, were isolated from a broiler duck farm and from carcasses of Baikal teals in Donglim Lake in South Korea. Two HPAI H5N1 viruses, A/chicken/Kr/IS/2006 (H5N1) (clade 2.2) [IS] and A/mandarin duck/Kr/PSC24–24/2010 (H5N1) (clade 2.3.2.1) [PSC24–24], were isolated on a chicken farm in 2006 and from a fecal sample collected from a wild bird habitat in South Korea in 2010, respectively. The viruses were propagated and titrated in specific pathogen–free (SPF) eggs and stored at –70°C until further use.

Experimental Design

The H5N8 isolates (Gochang1, Buan2, and Donglim3) were inoculated into 38 commercially obtained 2-week-old Pekin ducks to assess their pathogenicity and transmissibility. To assess pathogenicity in mallards, we inoculated 20 captured adult males and 21 adult females with Buan2 H5N8 virus, the main genotype circulating in South Korea, or with 2 HPAI H5N1 viruses (IS and PSC24–24) circulating in South Korea in 2006 and 2010. We also assessed H5N8 pathogenicity in 2 captured adult male Baikal teals.

To test pathogenicity, we intranasally inoculated each bird with 0.1 mL of each isolate containing the 50% egg infective dose ($10^{6.5}$ virions). After 8 h, 3 domestic ducks and 2 mallards were co-housed with inoculated birds as a contact group. We inoculated a control group of 3 domestic ducks and 2 mallards with 0.1 mL of phosphate-buffered saline using the same route. We euthanized 3 domestic birds and 2 mallards from each group 3 days postinoculation (dpi) to estimate virus recovery from various tissues.

We collected swab samples from the oropharynx and cloaca of domestic birds on 1–7, 10, and 14 dpi and from wild birds on 1, 3, 5, 7, 10, and 14 dpi. After the birds died or were euthanized, we collected tissues aseptically from the brain, trachea, lung, kidney, spleen, heart, cecal tonsil, liver, leg muscle, intestine and pancreas, and proventriculus for virus recovery. The remaining birds were observed clinically for 14 days. For virus isolation, cells from each oropharyngeal and cloacal sample were suspended in 1 mL of maintenance medium with antibiotic drugs (Antibiotic-Antimycotic; Invitrogen, Carlsbad, CA, USA), and each tissue sample was homogenized in maintenance medium with antibiotic drugs to effect a wt/vol ratio of 10%. Samples were then centrifuged at 3,500 rpm for 5 min, and 0.1 mL of supernatant was titrated in chicken embryo fibroblast cells to determine the median tissue culture infective dose ($TCID_{50}$); virus growth was determined by observing the cytopathic effect. Virus titers were calculated as described (*18*) and the limit of virus detection was <1. We performed statistical analysis using the Student *t* test; $p<0.05$ was considered statistically significant.

Serologic Assays

We collected pre-inoculation serum samples from each bird; all were confirmed to be negative for H5 HA influenza A virus by the HI assay, using standard procedures (*19*). In the H5N8 inoculation group, pre-inoculation serum samples of mallards were positive for anti-influenza virus antibody (C-ELISA; AniGen AIV Ab ELISA Kit; BioNote, Suwon, Gyeonggi-do, South Korea) but seronegative for H5 HA. Serum samples were collected from surviving wild birds, mallards, Baikal teals, and domestic ducks on 14 dpi to measure the antibody response. All serum samples were treated with receptor-destroying enzyme to remove nonspecific inhibitors (*19*).

Results

Clinical Signs and Mortality Rates in Domestic Ducks and Wild Birds

To determine pathogenicity, we inoculated 2-week-old domestic ducks intranasally with H5N8 viruses in groups of 3. The H5N8 viruses were moderately pathogenic (0%–20% mortality rate), and there were no differences in the patho-

genicity of the 3 viruses tested. Domestic ducks inoculated with the Buan2 virus exhibited depression and neurologic signs beginning on 9 dpi; all 5 infected domestic ducks survived. Of 10 domestic ducks (in 2 groups of 5) infected with the Gochang1 and Donglim3 viruses, 1 duck died on 8 dpi and 1 died on 11 dpi, respectively. Symptoms of depression, severe weight loss (32% and 34%, respectively), cloudy eyes, and intermittent head shaking were observed before death (Table 1). In domestic ducks on 14 dpi, seroconversion rates for the Gochang1, Buan2, and Donglim3 treatment groups were 40% (5.5 ± 0.7 $\log_2$), 80% (7.5 ± 0.6 $\log_2$), and 60% (7.3 ± 0.6 $\log_2$), respectively. In contact-group domestic ducks, HI titers for the Gochang1 and Buan2 virus were 5 $\log_2$ and 6 $\log_2$, respectively, but none of the 3 birds in the Donglim3 group seroconverted (Table 1).

Mallards excrete abundant quantities of HPAI H5N1 virus without exhibiting clinical signs of disease (*10*,*20*). In this study, none of the mallards infected with H5N1 or H5N8 viruses died. They exhibited mild or no symptoms after inoculation with the H5N8 or H5N1 virus. In mallards that were inoculated with H5N8, or in those that were in contact with H5N8 virus–inoculated birds, HI titers were much higher than those observed for the 2 H5N1 viruses. Of 5 mallards inoculated with the H5N8 virus, 4 (80%) seroconverted and showed high titers (9.0 ± 0.8 $\log_2$), and 2 contact-group ducks seroconverted with relatively high titers (7.0 ± 2.8 $\log_2$). The HI titers of groups inoculated with IS and PSC24–24 H5N1 viruses were 5.0 ± 0.7 $\log_2$, and 4 $\log_2$, respectively. In the IS and PSC24–24 virus-contact groups, the seroconversion rates were 50% (5 $\log_2$) and 0%, respectively.

Unlike mallards, 1 of the Baikal teals inoculated with an H5N8 virus died suddenly on 3 dpi without clinical symptoms. The surviving Baikal teal seroconverted to H5N8 with a relatively high titer (6 $\log_2$) (Table 2).

Replication in and Transmission among Domestic Ducks

The phenotypes of 3 H5N8 viruses, which were observed in 2 genotypes (Buan2/Donglim3, and Gochang1), were evaluated in domestic ducks. We found significant differences in the viral shedding of 3 H5N8 viruses in domestic ducks between Buan2 and Donglim3 viruses on cloacal swab samples on 5 dpi ($p<0.05$). In tissues, Donglim3 viral titers from trachea and lung samples were significantly higher than Buan2 virus titers, whereas Buan2 viral titers from spleen samples were significantly higher than those of Donglim3 ($p<0.05$), according to the results of the Student *t* test. The virus was not detected in a control group of domestic ducks (data not shown) that were not inoculated. In the infected domestic ducks, Gochang1 was recovered from the oropharynx ($10^{1.3–4.4}$ $TCID_{50}$/0.1 mL) on 1–7 dpi and from the cloaca ($10^{0.6–3.6}$ $TCID_{50}$/0.1 mL) on 1–6 dpi. The Buan2 virus was re-isolated from the oropharynx ($10^{0.6–3.7}$ $TCID_{50}$/0.1 mL) on 1–10 dpi and from the cloaca ($10^{0.6–2.9}$ $TCID_{50}$/0.1 mL) on 1–5 dpi. Donglim3 was recovered from the oropharynx ($10^{1.1–4.5}$ $TCID_{50}$/0.1 mL) on 1–10 dpi and from the cloaca ($10^{0.6–3.4}$ $TCID_{50}$/0.1 mL) on 2–7 dpi (Figure). The H5N8 viruses were replicated systemically in, and re-isolated from, various tissues of domestic ducks with titers that varied from $10^{0.7}$ to $10^{7.6}$ $TCID_{50}$/0.1 mL.

Unlike the other 2 H5N8 viruses, Gochang1 replicated at low titers ($10^{1.6}$ $TCID_{50}$/0.1 mL) in brain and other tissues. Gochang1 and Donglim3 viruses were isolated from several tissues of a dead inoculated bird (online Table 3, http://wwwnc.cdc.gov/EID/article/21/2/14-1268-T3.htm).

In domestic contact ducks, all 3 H5N8 viruses were recovered in swab samples, indicating that the H5N8 viruses could have spread by contact. Gochang1 virus was recovered from the oropharynx ($10^{1.7–4.1}$ $TCID_{50}$/0.1 mL) on 3–7 dpi and from the cloaca ($10^{0.6–3.7}$ $TCID_{50}$/0.1 mL) on 2–7 dpi. The Buan2 virus was recovered from the oropharynx ($10^{1.6–4.3}$ $TCID_{50}$/0.1 mL) on 3–7 dpi and from the cloaca ($10^{0.6–2.2}$ $TCID_{50}$/0.1 mL) on 3–7 dpi. Likewise, Donglim3 virus was recovered from the oropharynx ($10^{0.6–4.0}$ $TCID_{50}$/0.1 mL) on 2–7 dpi and from the cloaca ($10^{0.6–4.9}$ $TCID_{50}$/0.1 mL) on 3–7 dpi.

Virus Replication in and Transmission among Wild Birds

The extent of replication and transmissibility of a virus in the host animal has a major influence on the magnitude

Table 1. Weight loss, illness and death rates, and HI titers of domestic ducks exposed to 3 strains of influenza (H5N8) virus*

Virus	Group	Illness†	Weight loss, %‡	Deaths (%)	HI titer§ ($\log_2$, mean±SD)
A/breeder duck/Kr/Gochang1/2014 (H5N8)	Inoculated	3/5	32	1/5 (20)	2/5 (5.5 ± 0.7)
	Contact¶	2/3	21	0/3	1/3 (5)
A/broiler duck/Kr/Buan2/2014 (H5N8)	Inoculated	2/5	28	0/5	4/5 (7.5 ± 0.6)
	Contact¶	1/3	26	0/3	1/3 (6)
A/Baikal teal/Kr/Donglim3/2014 (H5N8)	Inoculated	3/5	34	1/5 (20)	3/5 (7.3)
	Contact¶	2/3	28	0/3	0/3
Controls (no virus exposure)		0/3	–	0/3	0/3

*Unless indicated otherwise, data represent the number of affected animals/ animals in the group. Animals were inoculated by the intranasal route with $10^{6.5}$ egg-infective $dose_{50}$/0.1 mL of the selected viruses; HI, hemagglutination inhibition.
†Severe depression, cloudy eye, and intermittent head-shaking.
‡Weight loss was measured at 11 d postinoculation and is expressed as a percentage of the weight of preinoculation ducks.
§HI titer was assayed in serum samples taken at 14 d postinoculation. Data show the ratio of antibody-positive animals to the number of virus-inoculated animals.
¶Three uninoculated birds were co-housed with infected birds as a contact group 8 h after inoculation.

Table 2. Virus isolation from swab samples obtained from 2 Baikal teal ducks inoculated with influenza A/broilerduck/Kr/Buan2/2014 (H5N8) virus*

Virus titer, log_{10} $TCID_{50}$/0.1 mL (no. positive/no. inoculated)†																			
Oropharyngeal samples, days postinoculation									Cloacal samples, days postinoculation									
1	2	3	4	5	6	7	10	14	1	2	3	4	5	6	7	10	14	HI titer‡
4.5 (1/2)	4.2 (1/2)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/2)	1.2 (1/2)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	0 (0/1)	1/1

*Birds were inoculated with $10^{6.5}$ 50% egg-infective dose/0.1 mL of virus via the intranasal route, and oropharyngeal and cloacal swab samples were collected on the indicated day. No clinical signs of illness were observed in the birds; however, 1 bird died on day 3 post-inoculation; TCID, tissue culture infective dose; HI, hemagglutination inhibition.
†Virus was detected only in the bird that died.
‡HI titer was assayed in a serum sample taken at day 14 postinoculation; data shows the ratio of the number of antibody-positive animals to the number of virus-inoculated animals.

of outbreaks. To evaluate the pathogenicity of the Buan2 H5N8 virus in comparison to that of 2 H5N1 viruses (IS06 and PSC24–24), mallards were inoculated intranasally with the viruses. H5N8 virus was re-isolated from the oropharynx ($10^{1.0–3.4}$ $TCID_{50}$/0.1 mL) on 1–5 dpi and from the cloaca ($10^{2.7}$ $TCID_{50}$/0.1 mL) on 3 dpi. In the H5N1-infected groups, the viruses were recovered from the oropharynx on 1–3 dpi, ($10^{1.8–2.0}$ $TCID_{50}$/0.1 mL) but not from the cloaca. The titers of the IS06 and PSC24–24 H5N1 virus re-isolated from oropharyngeal samples were significantly lower than that of the H5N8 virus on 3 dpi ($p<0.01$) (Table 4, http://wwwnc.cdc.gov/EID/article/21/2/14-1268-T4.htm). To determine whether the HPAI viruses can be efficiently transmitted among mallards, we performed the virus isolation procedures using oropharyngeal and cloacal samples obtained from mallards in the contact groups. All 3 H5 viruses were recovered, but their shedding patterns varied. H5N8 virus was recovered from the oropharynx ($10^{2.2–2.5}$ $TCID_{50}$/0.1 mL) on 3–5 dpi and from the cloaca ($10^{0.6}$ $TCID_{50}$/0.1 mL) on 3 dpi. However, the 2 H5N1 viruses could only be re-isolated from the oropharynx at low titers ($10^{1.8–2.0}$ $TCID_{50}$/0.1 mL) (Table 4).

The H5N8 virus was isolated from tissues collected from euthanized mallards on 3 dpi. It was replicated systemically in the trachea, muscle, proventriculus, intestine (pancreas), cecal tonsil, lung, kidney, and heart, and was present at low titers ($10^{1.8–2.6}$ $TCID_{50}$/0.1 mL). In infected birds, H5N1 IS06 and PSC24–24 viruses did not replicate in any of the tissues tested. H5N1 viruses were not detected in mallards in uninoculated control groups (online Table 3).

Two Baikal teal ducks were inoculated with influenza A/broiler duck/Kr/Buan2/2014 (H5N8). One of the ducks died on 3 dpi. H5N8 virus was re-isolated from its oropharyngeal sample ($10^{4.2–4.5}$ $TCID_{50}$/0.1 mL) on 1 and 2 dpi

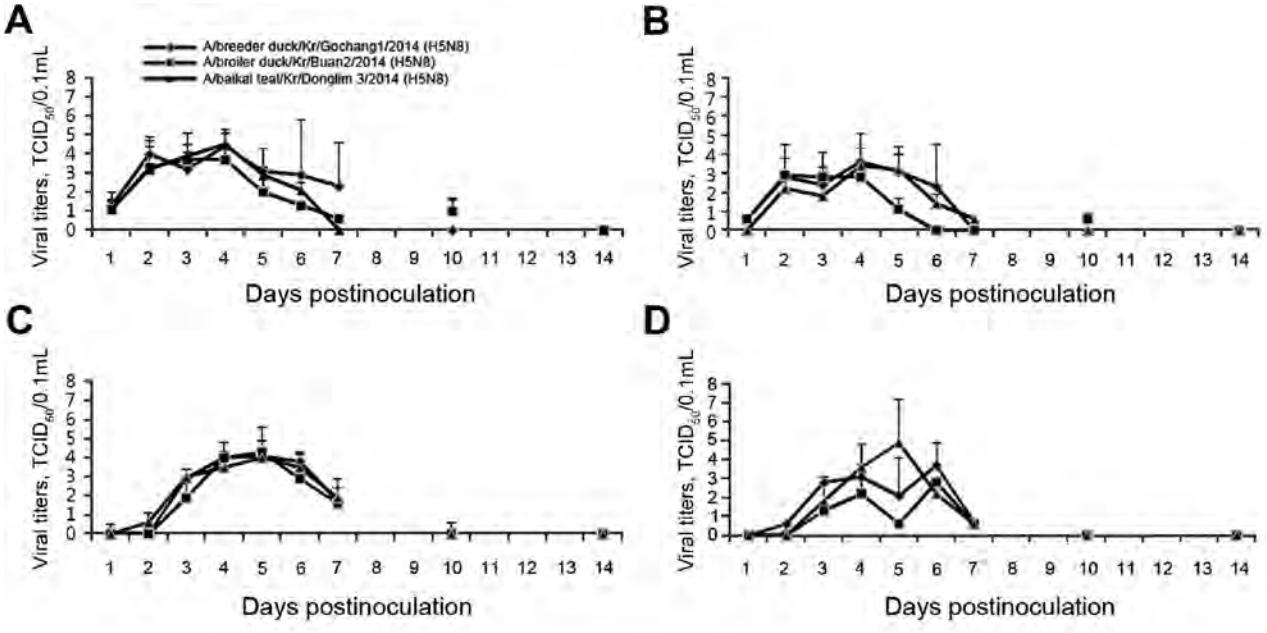

Figure. Virus isolation from oropharyngeal (OP) or cloacal (CL) swab samples collected from domestic ducks exposed to influenza viruses by inoculation or contact with infected ducks. Nine ducks were intranasally inoculated with $10^{6.5}$ egg infectious dose titer of A/breeder ducks/Kr/Gochang1/2014 (H5N8), A/broiler duck/Kr/Buan2/2014 (H5N8), or A/Baikal teal/Kr/Donglim3/2014 (H5N8) viruses (A and B). Six domestic ducks that were not inoculated were co-housed with 3 contact groups (2 in each group) of infected ducks (C and D). $TCID_{50}$, 50% tissue culture infectious dose. Error bars indicate SD.

and from the cloacal sample ($10^{1.2}$ $TCID_{50}$/0.1 mL) on 2 dpi (Table 2). The virus had replicated efficiently in the tissues of the bird by 3 dpi, and investigation after its death showed titers of $10^{2.4}$ $TCID_{50}$/0.1 mL in the trachea, $10^{1.3}$ in muscle, $10^{8.4}$ in liver, $10^{2.6}$ in the proventriculus, $10^{3.2}$ in the intestine (pancreas), $10^{2.6}$ in the spleen, $10^{2.8}$ in the cecal tonsil, $10^{2.4}$ in the lung, $10^{1.6}$ in the heart, and $10^{3.2}$ in the kidney (data not shown). No clinical signs of disease were observed in either duck.

Discussion

In a previous study, we reported the first influenza outbreak in South Korea in poultry and wild birds caused by a novel reassortant H5N8 virus in 2014 (*13*). The virus was composed of 2 distinct genogroups (*17*), and the most affected poultry species was the domestic duck at a 75.5% infection rate. Many H5N8 isolates were obtained from dead wild birds, but some were obtained from live wild birds, including mallards. In this study, we evaluated the pathogenicity of these novel reassortant HPAI H5N8 viruses in wild mallard and young domestic ducks.

The clade 2.3.4, which is the major genotype in China, continues to evolve as subclades, resulting thus far in 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.4.4, 2.3.4.5, and 2.3.4.6 (*21*). In addition, clade 2.3.4 appears in various NA subtypes such as H5N5, H5N8, and H5N2 (*22*,*23*). The pathogenicity of H5 viruses with different NA subtypes has been evaluated. A mortality rate of 50% in 4-week-old ducks was attributed to H5N5 viruses isolated from ducks in live bird markets of China in 2008 (*24*). The pathogenicity of H5N5 and H5N8 viruses isolated from poultry in China during 2009–2010 varied from mild to moderate among mallards (*16*). The pathogenicity of H5N8 viruses isolated from domestic ducks in eastern China in 2013, which was similar to that of the Gochang1 virus described here, has been evaluated only in chickens and mice (*16*).

We selected 3 H5N8 viruses belonging to 2 distinct genotypes (*13*) from different host animals and evaluated their pathogenicity in 2-week-old domestic ducks. There were no differences in the pathogenicity of the 3 H5N8 viruses found in South Korea, and they were less pathogenic (0%–20% mortality rate) than previous H5N1 viruses in South Korea that caused outbreaks in 2008 and 2010 (50%–100% mortality rate) (*10*,*12*,*25*). However, H5N8 viral shedding by domestic ducks was much greater ($10^{4.5}$ $TCID_{50}$/0.1 mL) in both oropharyngeal and cloacal swab samples than shedding of H5N1 viruses found in South Korea during 2008 ($10^{3.8}$ $TCID_{50}$/0.1 mL) and 2010 ($10^{2.8}$ $TCID_{50}$/0.1 mL) (*12*,*25*). The ability of these novel reassortant H5N8 viruses to replicate efficiently in the respiratory and intestinal tracts without killing the infected ducks enables them to circulate within the duck population and increases the possibility of transmission on poultry farms. Indeed, clinical signs and death were rare during the 2014 H5N8 outbreak in South Korea, except for a drop in egg production at duck breeder farms. The results of this study suggest that domestic ducks may be silent carriers of novel reassortant H5N8 viruses, which may make it difficult to detect these viruses in domestic duck farms or live bird markets. Moreover, the efficient replication, high seroconversion, and shedding of relatively high titers in the contact groups suggest that the H5N8 virus was efficiently transmitted among ducks. Therefore, active surveillance designed to detect infection, especially in domestic ducks, should be enforced on farms and at live bird markets.

Tang et al. (*26*) suggested that certain amino acid substitutions (Q→L at position 9) and 1 basic amino acid deletion within the HA cleavage site may be consequential for H5N1 pathogenicity in ducks. As mentioned previous report (*17*), each of the 3 H5N8 viruses in our study showed L at position 9 with a deletion at position 4 of the HA cleavage site, whereas H5N1 viruses circulating in South Korea in 2008 and 2010 showed Q at position 9 with a deletion at the same position (*5*,*12*). However, previous H5N1 viruses were reported as more pathogenic (50%–100%) than H5N8 viruses (0%–20%) in studies that used domestic ducks as hosts (*10*,*12*,*25*). Our results suggest that molecular factors other than amino acid substitutions in the HA cleavage site may be involved in the pathogenicity of these viruses in domestic ducks. Further study is required to identify the molecular factors determining virus pathogenicity in domestic ducks.

Some of the H5N1 and H5N8 viruses were isolated from apparently healthy mallards captured in South Korea during the 2010–11 (*5*) and 2014 outbreaks. We selected 1 H5N8 and 2 H5N1 viruses to evaluate their pathogenicity and transmission in mallards. Although severe morbidity or mortality rates were not observed in the mallards inoculated with H5N8 or H5N1 viruses or in those housed with inoculated ducks, viral shedding and replication in tissues were higher and the duration of viral shedding was longer in mallards infected with H5N8 virus than in those infected with H5N1 virus. In this study, the significant shedding of H5N8 viruses ($p<0.05$) by mallards is consistent with the hypothesis that mallards may be long distance vectors of these viruses, as was observed for HPAI H5N1 (*20*,*27*). Moreover, a significant difference in the viral shedding was found between domestic ducks and mallards: the Buan2 viral titers in domestic ducks were significantly higher than those in mallards on oropharyngeal and cloacal swabs on 2 and 4 dpi. ($p < 0.05$). Our results suggest that the novel reassortant H5N8 virus replicated more efficiently than H5N1 viruses in mallards and that efficient horizontal transmission occurred, resulting in the transmission of influenza virus between

and from the cloacal sample ($10^{1.2}$ $TCID_{50}$/0.1 mL) on 2 dpi (Table 2). The virus had replicated efficiently in the tissues of the bird by 3 dpi, and investigation after its death showed titers of $10^{2.4}$ $TCID_{50}$/0.1 mL in the trachea, $10^{1.3}$ in muscle, $10^{8.4}$ in liver, $10^{2.6}$ in the proventriculus, $10^{3.2}$ in the intestine (pancreas), $10^{2.6}$ in the spleen, $10^{2.8}$ in the cecal tonsil, $10^{2.4}$ in the lung, $10^{1.6}$ in the heart, and $10^{3.2}$ in the kidney (data not shown). No clinical signs of disease were observed in either duck.

Discussion

In a previous study, we reported the first influenza outbreak in South Korea in poultry and wild birds caused by a novel reassortant H5N8 virus in 2014 (*13*). The virus was composed of 2 distinct genogroups (*17*), and the most affected poultry species was the domestic duck at a 75.5% infection rate. Many H5N8 isolates were obtained from dead wild birds, but some were obtained from live wild birds, including mallards. In this study, we evaluated the pathogenicity of these novel reassortant HPAI H5N8 viruses in wild mallard and young domestic ducks.

The clade 2.3.4, which is the major genotype in China, continues to evolve as subclades, resulting thus far in 2.3.4.1, 2.3.4.2, 2.3.4.3, 2.3.4.4, 2.3.4.5, and 2.3.4.6 (*21*). In addition, clade 2.3.4 appears in various NA subtypes such as H5N5, H5N8, and H5N2 (*22*,*23*). The pathogenicity of H5 viruses with different NA subtypes has been evaluated. A mortality rate of 50% in 4-week-old ducks was attributed to H5N5 viruses isolated from ducks in live bird markets of China in 2008 (*24*). The pathogenicity of H5N5 and H5N8 viruses isolated from poultry in China during 2009–2010 varied from mild to moderate among mallards (*16*). The pathogenicity of H5N8 viruses isolated from domestic ducks in eastern China in 2013, which was similar to that of the Gochang1 virus described here, has been evaluated only in chickens and mice (*16*).

We selected 3 H5N8 viruses belonging to 2 distinct genotypes (*13*) from different host animals and evaluated their pathogenicity in 2-week-old domestic ducks. There were no differences in the pathogenicity of the 3 H5N8 viruses found in South Korea, and they were less pathogenic (0%–20% mortality rate) than previous H5N1 viruses in South Korea that caused outbreaks in 2008 and 2010 (50%–100% mortality rate) (*10*,*12*,*25*). However, H5N8 viral shedding by domestic ducks was much greater ($10^{4.5}$ $TCID_{50}$/0.1 mL) in both oropharyngeal and cloacal swab samples than shedding of H5N1 viruses found in South Korea during 2008 ($10^{3.8}$ $TCID_{50}$/0.1 mL) and 2010 ($10^{2.8}$ $TCID_{50}$/0.1 mL) (*12*,*25*). The ability of these novel reassortant H5N8 viruses to replicate efficiently in the respiratory and intestinal tracts without killing the infected ducks enables them to circulate within the duck population and increases the possibility of transmission on poultry farms. Indeed, clinical signs and death were rare during the 2014 H5N8 outbreak in South Korea, except for a drop in egg production at duck breeder farms. The results of this study suggest that domestic ducks may be silent carriers of novel reassortant H5N8 viruses, which may make it difficult to detect these viruses in domestic duck farms or live bird markets. Moreover, the efficient replication, high seroconversion, and shedding of relatively high titers in the contact groups suggest that the H5N8 virus was efficiently transmitted among ducks. Therefore, active surveillance designed to detect infection, especially in domestic ducks, should be enforced on farms and at live bird markets.

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Vesicular Stomatitis Virus–Based Vaccines against Lassa and Ebola Viruses

Andrea Marzi, Friederike Feldmann,Thomas W. Geisbert, Heinz Feldmann, David Safronetz

We demonstrated that previous vaccination with a vesicular stomatitis virus (VSV)–based Lassa virus vaccine does not alter protective efficacy of subsequent vaccination with a VSV-based Ebola virus vaccine. These findings demonstrate the utility of VSV-based vaccines against divergent viral pathogens, even when preexisting immunity to the vaccine vector is present.

Viral hemorrhagic fevers (VHFs) are caused by a group of genetically distinct zoonotic viruses, which include ≥4 virus families (*Filoviridae*, *Arenaviridae*, *Bunyaviridae*, and *Flaviviridae*). Because of major and often highly publicized outbreaks, the most recognized VHF agents are Ebola virus (EBOV), Marburg virus (MARV), and Lassa virus (LASV). However, there are many other prominent etiologic agents of VHFs that (when infection numbers are combined) result in hundreds of thousands of infections annually, which cause a major burden to public health care systems worldwide (*1*).

Illness and death associated with these pathogens, combined with the threat of intentional release, has led to intensive research efforts to develop rapid-acting, safe, and effective medical countermeasures to control the spread of VHFs. Because of nonspecific clinical onset; rapid progression to severe disease; uncertain pathophysiology of disease; and high viral loads in blood, secretions, or excretions of affected patients that can result in human-to-human transmission, the ideal countermeasure remains prophylactic vaccination. Several vaccine platforms have shown efficacy against individual VHF pathogens, many of which are based on similar platforms (*1*). Among the most successful VHF vaccine candidates are recombinant, replication-competent vesicular stomatitis virus (VSV)–based vectors, which in animal models have proven highly effective in preventing lethal disease after challenge with a variety of high-consequence viral pathogens, including, but not limited to, EBOV, MARV, and LASV, as well as Andes (ANDV) and Nipah viruses (*2*–*5*).

Although protective efficacy of individual vaccination in challenge experiments is evident, few studies have addressed the effect of sequential, long-term, vaccination strategies with 1 vaccine platform against multiple VHFs, including the VSV-based strategy. One study showed that a single vaccination, using a vaccine made from multiple VSV filovirus vaccines, affords protection against an otherwise lethal challenge with distinct EBOV species and a MARV isolate (*6*). In addition, multiple consecutive vaccinations in a short period (≤14 days) with filovirus-specific VSV vaccines have been shown to elicit protective, possibly cross-reactive, immune responses in nonhuman primates (NHPs) (*6*,*7*). However, it remains unclear whether sequential vaccinations within a population that has mounted a complete humoral immune response (≥90 days) after an initial vaccination with a VSV-based vaccine would elicit a robust and protective immune response after vaccination with a second VSV-based vaccine.

Although this question was previously overlooked because of the restricted geographic distribution of many etiologic agents of VHF for which VSV-based vaccines have been tested, the emergence of EBOV in countries in West Africa to which LASV is endemic has heightened concerns of use and efficacy of 1 vaccine platform against multiple agents of VHF (*8*). The purpose of this study was to determine if previous vaccination with a VSV-based LASV vaccine would reduce the efficacy of subsequent vaccination with the VSV-based EBOV vaccine (Figure).

The Study

This study was conducted in accordance with a protocol approved by an Institutional Animal Care and Use Committee of the National Institutes of Health. All laboratory work with potentially infectious materials was conducted in a Biosafety Level 4 facility at the Rocky Mountain Laboratories (Division of Intramural Research/National Institute of Allergy and Infectious Diseases/National Institutes of Health).

Three cynomologus macaques were vaccinated with 1 dose of 10^7 PFU of VSVΔG/LASVGPC, a live-attenuated, recombinant viral vaccine in which the VSV surface glycoprotein has been replaced with those of LASV, by intramuscular injection as described (*4*). Another age-matched control animal was vaccinated with an irrelevant VSV-based vaccine (VSVΔG/ANDVGPC) (*5*). At 28 days postvaccination, the 4 NHPs were challenged with a lethal dose of LASV (10^4 50% tissue culture infectious doses [$TCID_{50}$s])

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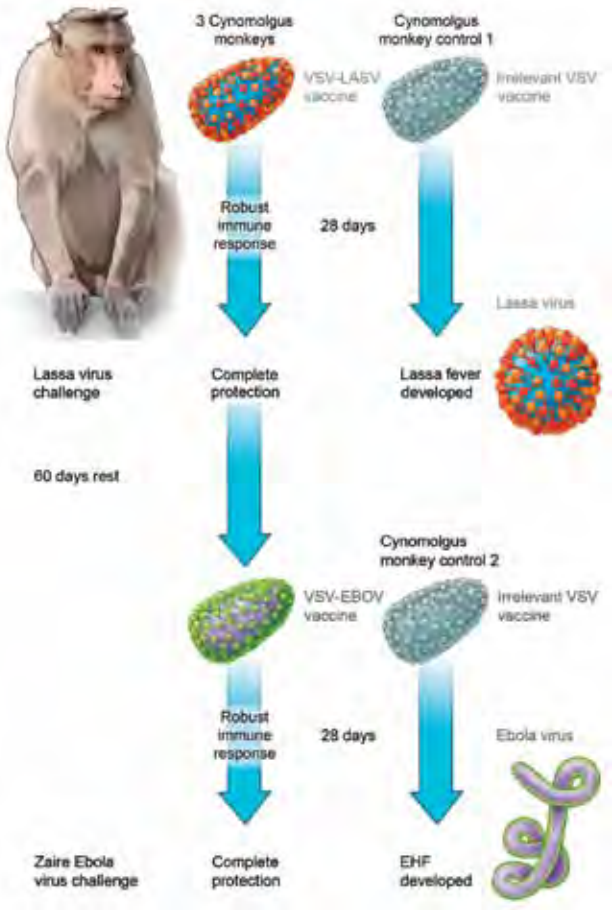

Figure. Effect of sequential vaccination with recombinant vesicular stomatitis virus (VSV)–based vaccines on protective efficacy afforded by each vaccine in nonhuman primates. Vaccination with a VSV-based Lassa virus vaccine encoding the Lassa virus glycoproteins provides complete and possibly sterile immunity against a lethal Lassa virus (LASV) challenge. Approximately 90 days after receiving the initial VSV–Lassa vaccine, animals were vaccinated with a VSV-based Ebola virus (EBOV) vaccine. Although we observed a robust VSV-specific immune response, the VSV–Ebola virus vaccine provided complete and possibly sterile immunity against a lethal Zaire Ebola virus challenge. EHF, Ebola hemorrhagic fever.

(*9*). The control animal showed signs of Lassa fever 7–10 days postinoculation and was euthanized 13 days postchallenge because of severity of disease. Classic indicators of Lassa fever, including decreased total protein and albumin; increased serum levels of alanine aminotransferase, aspartate aminotransferase, amylase, blood urea nitrogen, and alkaline phosphatase; and hematologic abnormalities, including thrombocytopenia and lymphopenia, were apparent in this animal.

Virus isolation conducted for select tissue samples showed LASV titers of 5–7 $\log_{10}$ $TCID_{50}$/g of tissue; blood samples collected on day 10 and at the time of euthanasia (5 and 6.25 $\log_{10}$ $TCID_{50}$/mL, respectively) showed viremia. In contrast, the 3 animals vaccinated with VSVΔG/LASVGPC resisted lethal LASV challenge and did not demonstrate any clinical signs of disease or any hematologic or biochemical indicators of LASV infection. At no point in the study was virus found in blood samples collected regularly from these 3 animals, even when tested by sensitive reverse transcription PCRs. An ELISA with serum samples collected 45 days postchallenge demonstrated equivocal antibody titers (100) against a recombinant LASV nucleocapsid protein in 1 NHP. The other 2 animals did not show seroconversion, which suggested that vaccination caused nearly sterile immunity against LASV (Table).

Approximately 90 days after the original vaccination with VSVΔG/LASVGPC, the 3 NHPs were vaccinated with a single dose of 10^7 PFU of VSVΔG/EBOVGP by intramuscular injection as described (*3*). An additional NHP was vaccinated with a control vaccine as outlined above and served as the inoculation control. At the time of vaccination, the 3 macaques had a robust VSV-specific antibody response with titers of 25,600, as determined by a whole virus ELISA (Table). Despite this finding, the 3 animals that received the VSV-based EBOV vaccine mounted an efficient response to the EBOV glycoprotein (Table) and were completely protected when challenged 28 days later with a lethal dose of EBOV (10^3 PFU) (*10*). Postchallenge, the 3 NHPs did not show any clinical signs of disease. Hematologic and serum biochemistry values remained constant throughout the study, and virus was not found in blood samples collected regularly and tested by using real-time reverse transcription PCR.

In contrast, severe EBOV hemorrhagic fever developed in the control animal, which was characterized by increased serum concentrations of alkaline phosphatase, aspartate aminotransferase and alanine aminotransferase; thrombocytopenia; and viremia (≥7 $\log_{10}$ $TCID_{50}$/mL whole blood) beginning 3–6 days postchallenge. This animal was euthanized 7 days postchallenge, and titration of selected tissue samples showed EBOV titers of >9 $\log_{10}$ $TCID_{50}$/g tissue. An ELISA conducted at the conclusion of the study (42 days post–EBOV challenge) showed increased antibody responses to VSV (titers =102,400) and seroconversion to the EBOV viral protein 40 antigen (titers 1,600–6,400) in the 3 surviving NHPs (Table), which is consistent with published results (*10*).

Conclusions

Because of the remote locations where VHF agents are present, an overall shortage of health care professionals and clinics in these locations, and mobility of human populations, any vaccine against these pathogens would ideally need to elicit a protective immune response after a single vaccination. For this reason, replication-competent viral vectors are considered leading VHF vaccine candidates.

Table. Serologic responses in 3 nonhuman primates sequentially vaccinated with 2 VSV-based VHF vaccines*

	Lassa virus challenge study				Ebola virus challenge study			
	Prechallenge	Postchallenge			Prechallenge	Postchallenge		
Nonhuman primate no.	LASV GPC	LASV GPC	LASV NP	VSV	EBOV GP	EBOV GP	EBOV VP40	VSV
1	ND	ND	100	25,600	6,400	25,600	1,600	102,400
2	ND	ND	<100	25,600	6,400	25,600	6,400	102,400
3	ND	ND	<100	25,600	6,400	25,600	6,400	102,400

*VSV, vesicular stomatitis virus; VHF, viral hemorrhagic fever; LASV, Lassa virus; GPC, glycoprotein precursor; NP, nucleocapsid protein; EBOV, Ebola virus; GP, glycoprotein; VP40 viral protein 40; ND, not determined. Titers are indicated as reciprocal endpoint dilutions from ELISAs for recombinant antigens (LASV NP, EBOV GP, and VP40), or whole virus preparations (VSV).

As the VSVΔG/EBOVGP vaccine heads toward clinical trials, it is necessary to clarify the potential limitations of using the VSV platform against multiple VHF agents. A major drawback for many viral vector platforms is preexisting immunity against the vector itself, which can decrease or nullify the essential protective immune response, which results in vaccine failure. Design of the VSV-based vaccines, which encode and express glycoproteins from various pathogens without its own glycoprotein (*11*,*12*), suggest that preexisting immunity would not influence protective efficacy of individual vaccinations (*12*). However, until now, this possibility has not been examined in disease models. Results of this study demonstrate that multiple VSV vaccines can be used in a population without any deleterious effect on overall protective efficacy.

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Use of Insecticide-Treated House Screens to Reduce Infestations of Dengue Virus Vectors, Mexico

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Dengue prevention efforts rely on control of virus vectors. We investigated use of insecticide-treated screens permanently affixed to windows and doors in Mexico and found that the screens significantly reduced infestations of *Aedes aegypti* mosquitoes in treated houses. Our findings demonstrate the value of this method for dengue virus vector control.

Vector control is the primary method for prevention and control of the increasingly frequent dengue outbreaks that threaten more than half the global human population (*1*). Existing approaches target breeding sites or attack adult mosquitoes by insecticide space-spraying, but these methods, at best, offer only immediate solutions and are rarely effective or sustainable for the long term (*2*). Methods that target the largely endophilic adult female *Aedes aegypti* mosquito vectors within buildings where they rest and bloodfeed have greater potential for sustained results and acceptance at the community level. One such method, long-lasting insecticidal-net (LLIN) curtains hung at windows or doors, can greatly reduce vector populations at high coverage rates (*3*–*5*), but efforts are compromised when curtains remain open during daytime or when all house entry points cannot be protected (*6*,*7*). Fixed or permanent screens covering doors and windows could eliminate this problem. Mosquito-proofing of houses is effective in malaria control (*8*), and reduced risk for dengue has been associated with the use of untreated (*9*) and insecticide-treated (*3*,*10*) screens.

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The Study

During 2011–2013, in the city of Acapulco in Guerrero state, Mexico (Figure 1), an area of consistently high dengue transmission (http://www.epidemiologia.salud.gob.mx/dgae/panodengue/intd_dengue.html), we investigated the effect on vector infestations of permanently mounted, insecticide-treated screens fitted to door and windows of residential houses. The screens (Duranet, Clarke Mosquito Control, Roselle, IL, USA) were made of 0.55% wt/wt α-cypermethrin–treated nonflammable polyethylene netting (145 denier; mesh = 132 holes/in^2); the design is approved by the World Health Organization (WHO) Pesticide Evaluation Scheme (http://www.who.int/whopes/en/).

We used a cluster-randomized sampling design constructed on the basis of earlier studies (*4*–*6*,*11*) to select 20 clusters (10 treatment, 10 control; 100 households/cluster) from a possible 30 clusters by using digital maps (Google Earth software; Google Inc., Mountain View, CA, USA) (Figure 1). Sample size was determined by using a 2-level hierarchical model to achieve 80% power at a 5% level of significance. Thus, for a negative binomial distribution with a dispersion coefficient of 0.02 and intracluster coefficient of 0.05, a minimum of 8.9 clusters/arm were required. Written informed consent was obtained from participating households; the WHO Ethical Review Committee (WHO reference no. 2010/82951-0, unit reference no. A90297) and Guerrero State Ministry of Health granted ethical permission for the study.

Participating households in the treatment arm were instructed on LLIS maintenance during installation (April–December 2012). Control houses received no treatment. Five entomologic surveys of randomly selected houses were conducted: before intervention (March 2011, September 2011, March 2012) and at 5 and 12 months after intervention (September 2012, March 2013; wet and dry seasons, respectively). Before intervention, 32 houses per cluster were sampled at each survey; after intervention, 210 houses from treated clusters and 302 from control clusters

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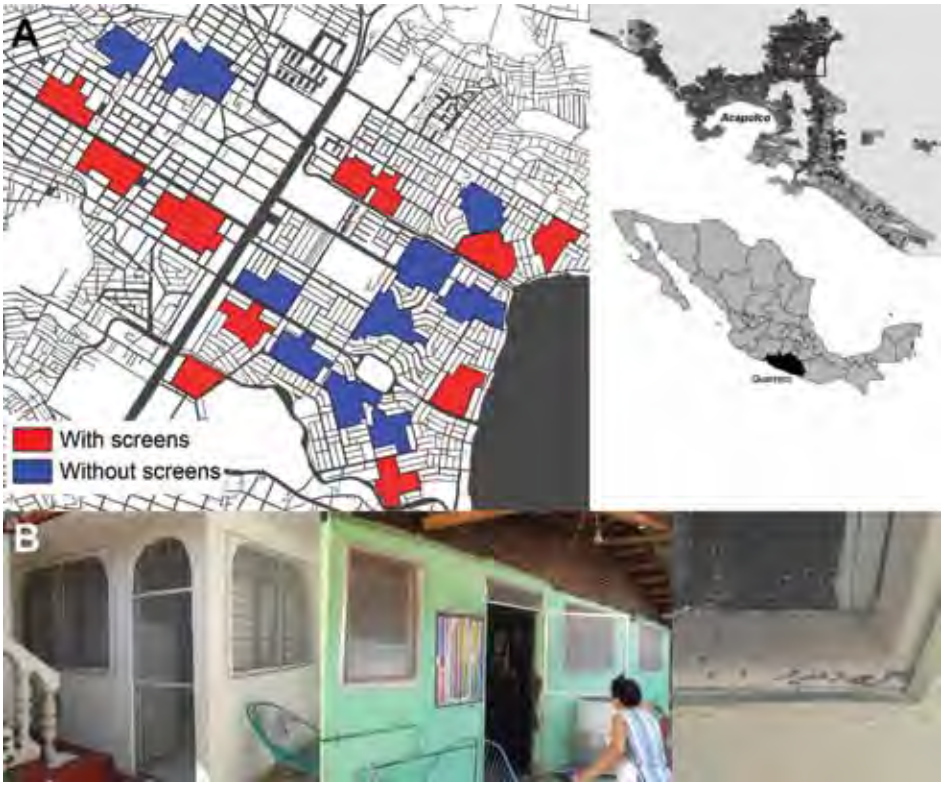

Figure 1. Area of study of long-lasting insecticide-treated screens in Acapulco, Mexico, March 2011–March 2013. A) Locations of clusters in the neighborhoods of Ciudad Renacimiento and Zapata, showing areas with (red) and without (blue) screens. Insets show location of study area (black box) in Acapulco and Guerrero state (black shading) in Mexico. B) Photographs of screens mounted on aluminum frames and fixed to windows and external doors of treated houses in 2012. The insects visible in the right photograph are dead house flies.

were sampled in September 2012 and 311 houses from treated and 320 from control clusters in March 2013.

Indoor resting adult mosquitoes were collected by using modified CDC backpack aspirators (John W. Hock Co., Gainesville, FL, USA) from all houses in a cluster on the same day during 9 AM–3 PM. Indices for *Ae. aegypti* mosquitoes (the only *Aedes* species found) were calculated to quantify house infestation (percent of all houses positive) and infestation density (numbers per infested house) for all mosquitoes, all females, all blood-fed females, and males.

For presence–absence data, we performed logistic regression models with a single predictor variable identifying houses with LLIS and control houses (coded as 1 and 0, respectively) and accounting for each house membership in a given sampling cluster (cluster-robust SE calculation). Odds ratios (ORs) and 95% CIs indicating the effect of LLIS on each entomologic indicator were calculated. Overdispersed index data were compared between arms by using the Mann-Whitney U test. The effect of treatment on each metric was analyzed by negative-binomial regression using, as with the logistic models, treatment as the sole predictor variable (1 and 0 coding). Negative binomial models also accounted for membership of a house in a sampling cluster (cluster-robust SE calculation). ORs and incidence rate ratios (IRRs) were calculated with 95% CIs; significance was set at $p<0.05$. Analyses were performed by using Stata 12.0 (StataCorp, College Station, TX, USA).

Before intervention, indices were similar for both study arms on all sampling dates. House infestation rates (Figure 2, panels A–D) and mosquito densities (Figure 2, panels E–H) followed seasonal patterns (2-sample Wilcoxon rank-sum test for all treatment–control comparisons, $|z|<1.0$; $p>0.1$). At 5 months postintervention, significantly fewer treated than control houses were infested with *Ae. aegypti* adult female mosquitoes (OR 0.38, 95% CI 0.21–0.69), blood-fed females (OR 0.36, 95% CI 0.21–0.60), and males (OR 0.39, 95% CI 0.19–0.77). A significant effect was still seen at 12 months for adult females (OR 0.41, 95% CI 0.25–0.68) and males (OR 0.41, 95% CI 0.27–0.64) but not for blood-fed females (OR 0.51, 95% CI 0.24–1.05). Analyses of infestation density showed similar trends, with significantly fewer *Ae. aegypti* mosquitoes found in treated than in control houses: adult females at 5 (IRR 0.37, 95% CI 0.27–0.49) and 12 (IRR 0.40, 95% CI 0.23–0.70) months postintervention, males at 5 (IRR 0.39, 95% CI 0.28–0.54) and 12 (IRR = 0.49, 95%CI 0.33–0.72) months postintervention, and blood-fed females at 5 (IRR 0.32, 95% CI 0.23–0.45) but not 12 (IRR 0.49, 95% CI 0.23–1.05) months postintervention.

A comparison of wet season data from treatment houses before (August 2011) and after (September 2012) intervention showed that significantly fewer females and blood-fed females were found postintervention (Wilcoxon matched pairs W = 30706, $z = 3.717$, and W = 20706,

z = 3.146; $p<0.05$ for both comparisons). However, the number of male mosquitoes did not change significantly (W = 20706, z = 1.385; $p>0.05$).

At 5 months postintervention, fewer LLIS-treated houses (33%) than control houses (56%) remained infested with female *Ae. aegypti* mosquitoes. Lower numbers of female mosquitoes were also found per infested house (0.54 ± 0.9) than per control house (1.39 ± 2.0); this effect was still detectable at 12 months postintervention (18%, 0.3 ± 0.8, vs. 35%, 0.7 ± 1.4).

Conclusions

In our study, the entomologic effect of LLIS was greater than that detected in a recent study of deltamethrin-treated window curtains (*12*), in which a 27% reduction of adult *Ae. aegypti* mosquitoes was only sustained for a short time after curtain installation. Other studies of insecticide-treated curtains in Latin America have reported entomologic effects by using immature stage indicators alone (*4*,*5*,*7*). Whether these reductions were sufficient to affect dengue transmission is unknown, and the overall effect on dengue infections remains to be evaluated.

Our results are encouraging in view of high levels of insecticide resistance in *Ae. aegypti* mosquitoes in Acapulco. Although resistance to α-cypermethrin has yet to be reported in Guerrero, high frequencies of mutations in the voltage-gated sodium channel gene, which is associated with pyrethroid resistance in *Ae. aegypti* mosquitoes, have been reported (*13*). If insecticide resistance began to reduce the efficacy of the method we describe, the screens could be treated with different insecticide classes.

We found the use of LLIS was a popular intervention, and perceived efficacy was reinforced by a reduction in other domestic pests (Figure 1) (*14*). The likely effects on

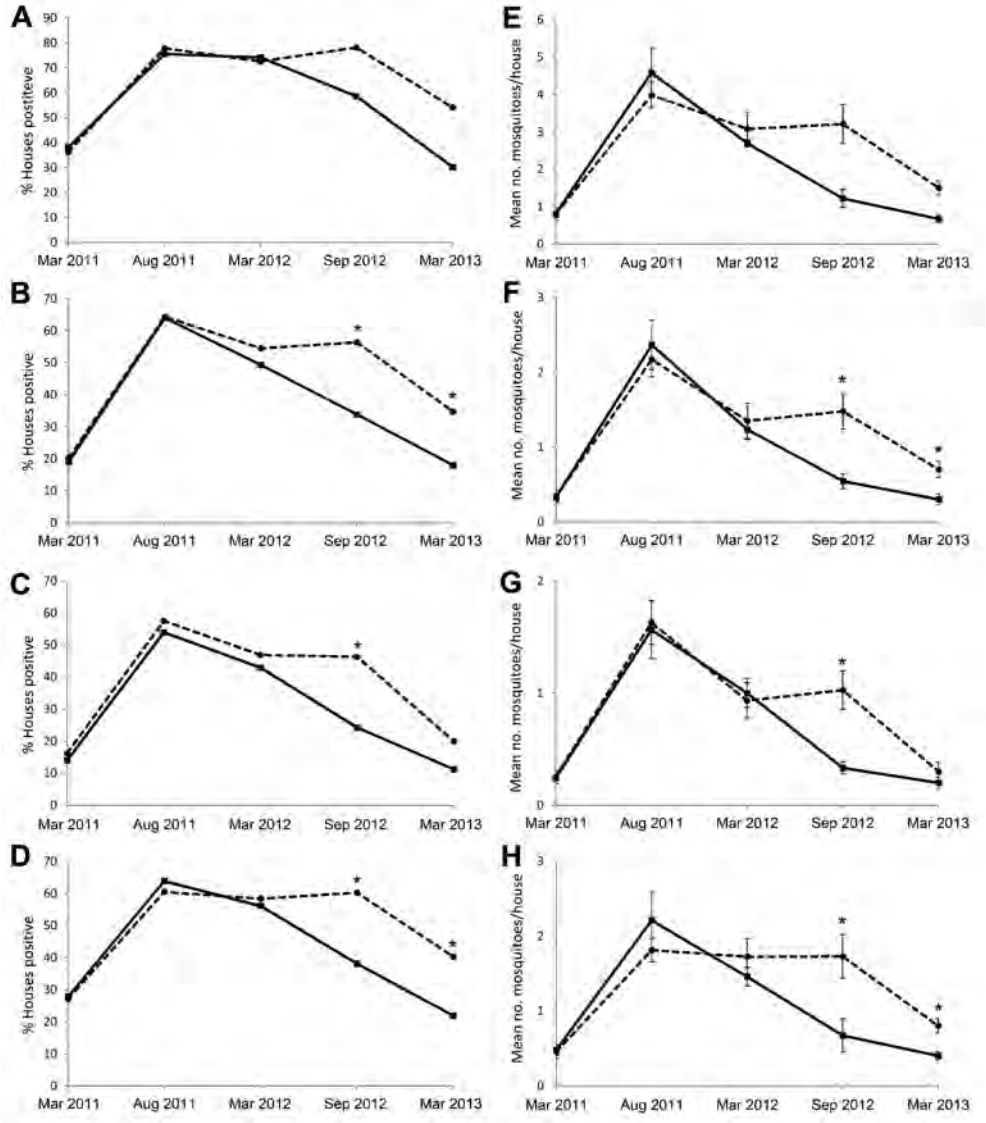

Figure 2. Infestation indices for adult *Aedes aegypti* mosquitoes in intervention (solid lines) and control (dashed lines) households before and after intervention in Acapulco, Mexico, as measured during dry (March) and wet (August–September) season cross-sectional surveys, 2011–2013. A–D) Vector prevalence: percentage of houses positive for A) all adults; B) all females; C) blood-fed females; D) males. E–H) Vector density: mean number per infested house for E) all adults; F) all females; G) blood-fed females; H) males. Error bars indicate SEs. Fitting of insecticide-treated window and door screens commenced during April 2012. Asterisks (*) denote dates when the index was significantly different between treated and control groups.

other peridomestic disease vectors could promote increased adoption of the intervention with additional cost benefits. The polyethylene netting was durable on windows; it was often damaged on the lower sections of doors (*14*) but readily repaired by reinforcement with metal mesh.

Dengue vector control programs using house screens are ongoing in selected cities in Mexico and Brazil. These results were obtained during an exploratory phase of that initiative. Stakeholders in other countries may also consider evaluating this novel approach for dengue vector control.

Acknowledgments

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Comparative Analysis of African Swine Fever Virus Genotypes and Serogroups

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African swine fever virus (ASFV) causes highly lethal hemorrhagic disease among pigs, and ASFV's extreme antigenic diversity hinders vaccine development. We show that p72 ASFV phylogenetic analysis does not accurately define ASFV hemadsorption inhibition assay serogroups. Thus, conventional ASFV genotyping cannot discriminate between viruses of different virulence or predict efficacy of a specific ASFV vaccine.

African swine fever (ASF) is a highly contagious hemorrhagic disease that causes high rates of death among domestic pigs. The disease is caused by ASF virus (ASFV), and the extreme antigenic diversity of the virus is one of the main obstacles to developing a safe and efficacious vaccine against ASF. Nevertheless, substantial progress has been made in understanding the pathogenesis of the disease and virus–host interactions (*1*,*2*). The ability to induce a protective immune response against ASFV has been demonstrated in numerous studies. Pigs that recover from ASF have long-term immunity to subsequent challenge with moderately virulent ASFV and related virulent viruses, but they rarely gain immunity to heterologous viruses (*3*–*6*). Because of these cross-protective responses, the antigenic diversity among naturally occurring ASFV isolates is of interest for ASFV vaccine development (*7*).

Researchers at the National Research Institute for Veterinary Virology and Microbiology (VNIIVViM) in Pokrov, Russia, have developed a classification of ASFV isolates based on a hemadsorption inhibition assay (HAI) with ASFV reference immune antisera. The results of a long-term study from VNIIVViM were used to serologically classify ASFV strains, isolates, and attenuated variants. Eight serogroups have been identified (serogroups 1–8), but more likely exist. In vaccine design and development, consideration should be given to the fact that viruses within a serogroup provide cross-protection from challenge with viruses of the same serogroup (*8*,*9*). VNIIVViM maintains a large and diverse collection of serologically grouped ASFV isolates that provides a unique resource for defining ASFV strain variability and establishing relationships of cross-protective immunity (*10*,*11*).

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Current genetic typing of ASFV isolates is based on nucleotide sequencing of the p72 capsid protein gene (B646L) and/or amplification of full-length polymorphisms of various genomic regions (*12*,*13*). During ASF outbreaks, these genotyping approaches can be used to identify the origin of viruses and quickly differentiate closely related strains. However, the correlation between currently established ASFV genotypes and viral cross-protection is not precisely clear (*6*). Thus, we examined the relationship of the established genotype distribution to HAI serologic classification.

The Study

Serologic classification was based on HAI results for ASFV strains maintained at VNIIVViM. These include isolates from disease outbreaks in Africa, Europe, the Caribbean, and, more recently, from the Russian/Trans-Caucasian epizootic and attenuated variants. In brief, swine red bone marrow cell culture was used for ASFV isolate amplification, and swine anti-ASFV serum and erythrocytes were subsequently added to the culture. ASFV isolates for which the hemadsorption phenomenon was inhibited by serum belonging to the same group within serogroups 1–8 were clustered into a homologous serogroup. Only ASFV hemadsorbing strains could be analyzed by this method, and some hemadsorbing ASFV isolates could not be placed into existing serogroups because HAI was not observed with available reference serum (*8*,*9*).

ASFV isolates from the depository at VNIIVViM were also classified by using a standard ASFV genotyping protocol previously published by Bastos et al. (*13*). In this method, the variable part of the p72 (B646L) gene was amplified by conventional PCR, and the amplicons were directly sequenced by using a 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. Chromatograms were manually edited and assembled by using CAP3 (http://pbil.univ-lyon1.fr/cap3.php). The nucleotide sequences of the ASFV isolates were deposited into GenBank (accession nos. KJ526354–KJ5263471).

Sequences determined at VNIIVViM were aligned with other publicly available ASFV sequences and analyzed by using minimum evolution; a rooted tree was constructed with MEGA 5.0 software (*14*) and edited with FigTree v1.4 (http://tree.bio.ed.ac.uk/) (Figure 1). The results of the ASFV genotyping are summarized in the Table.

[1]These authors contributed equally to this article.

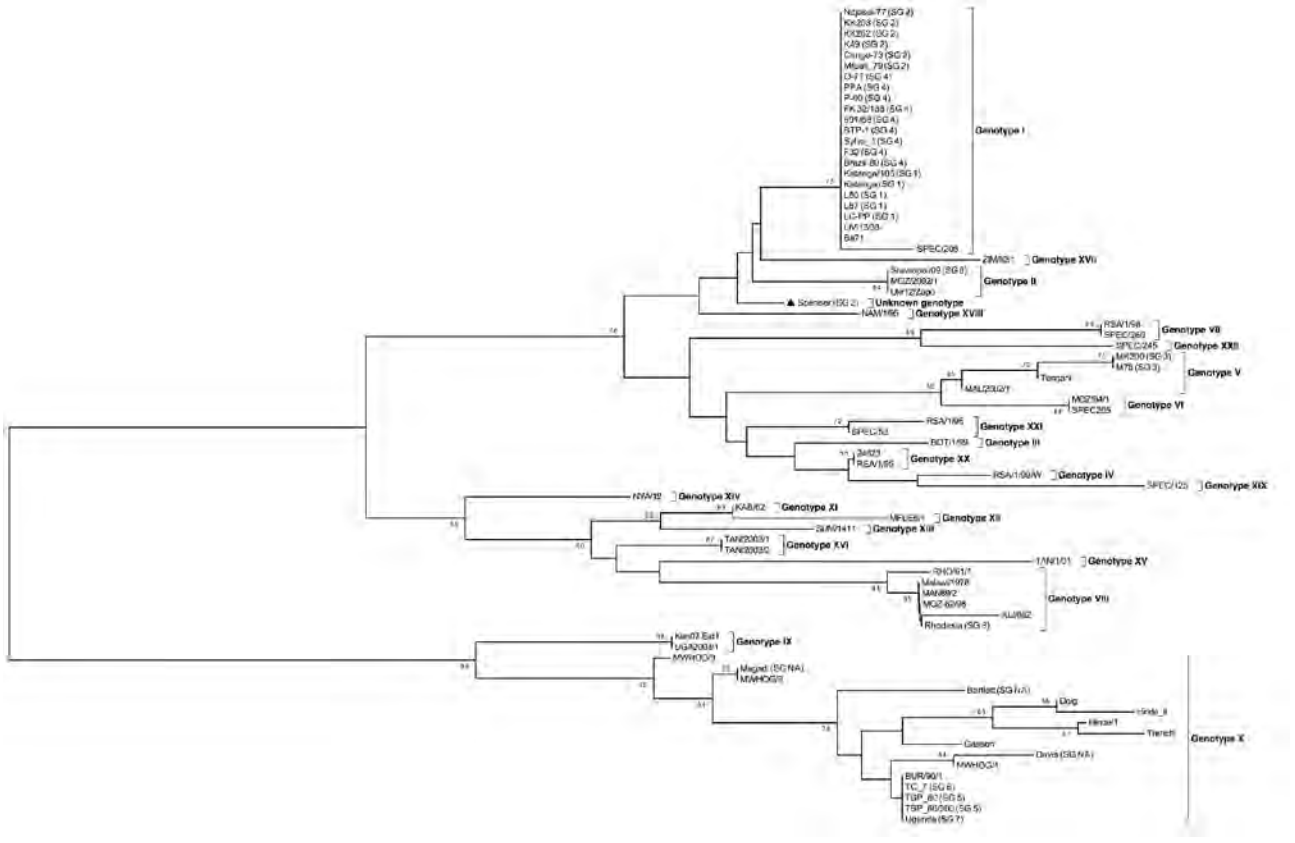

Figure 1. Phylogenetic tree of African swine fever virus (ASFV) isolates maintained in a collection at the National Research Institute for Veterinary Virology and Microbiology in Pokrov, Russia; the variable part of B646L gene relative to the 22 known p72 genotypes (labeled I-XXII) was used for analysis. The tree was reconstructed by using the minimum evolution method with 1,000 replicates. Bootstrap values >50 are shown above the nodes of the tree. Untyped ASFV isolate Spenser is indicated by a grey triangle.

Newly identified ASFV genotypes and known serogroups were mapped together so their geographic distribution in Africa and Europe (including the European part of the Russian Federation) could be visualized. The results (Figure 2) show that genotypic and serogroup diversity are greatest in a relatively limited area, mainly in southeastern Africa. In contrast, non-ASFV–endemic countries, where ASF outbreaks were caused by ASFV of a single genotype, exhibited low or no serogroup diversity.

Single genotype clades of ASFV were observed to contain viruses of multiple serogroups (Table). For example, ASFV isolates belonging to serogroups 1, 2, and 4 were specifically clustered within genotype I, and did not group with other genotypes. This indicates heterogeneity among ASFV strains previously isolated on the European continent.

We also found several serogroups of ASF viruses within genotype X. The ASFV isolates TSP80 (serogroup 5) and TS-7 (serogroup 6) were subsequently isolated from 1 field sample derived from a naturally infected pig in Tanzania. However both were genotype X viruses. Of note, 1 serogroup 2 isolate (Spenser) demonstrated a novel genotype within the p72 phylogenetic tree and relative to other serogroup 2 viruses (Figure 1), indicating that the p72 genotype, in addition to lacking serotype resolution, has potential to be incongruous relative to serogroup. Together, these data indicate that the antigenic heterogeneity of ASFV strains is not fully captured by using the standard genotyping approach.

Conclusions

The virus elements responsible for protective and cross-protective immune responses are not well known. Given the structural and genetic complexity of ASFV, it is likely that genes encoding different antigens will be more suited for virus typing. Substantial genetic variability can exist between strains and predominate in specific genomic regions, and it is these regions that may provide improved targets for genotyping. Our findings support that of a previous study that showed that geographic areas with ASFV of high genotypic and serotypic diversity

Table. ASFV isolates selected for inclusion in a study comparing ASFV genotypes and serogroups*

Isolate	Country of origin†	Year: Isolated from primary outbreak	Year: Isolate deposited‡	Year: Attenuated variant deposited‡	p72 genotype	GenBank accession no.	Serogroup
L57	Portugal	1957	1982	–	I	AF301537.1	1
L50	Portugal	NK	1983	–	I	AF301537.1	1
LC-PP	Portugal	–	1967	1967	I	AF301537.1	1
Katanga	Zaire (DRC)	NK	1978	–	I	KJ526355	1
Katanga/105	Zaire (DRC)	NK	1978	–	I	KJ671546	1
STP-1	Sao-Tome and Principe	1979	1979	–	I	KJ526371	4
P-60	Portugal	NK	1978	–	I	AF301539	4
F-32	France	1964	1969	–	I	KJ671547	4
FK-32/135	France	–	1973	1973	I	KJ526370	4
O-77	USSR (Ukraine)	1977	1977	–	I	KJ671544	4
Brasil-80	Brazil	1979	1980	–	I	KJ526367	4
691/88	Switzerland	NK	1989	–	I	KJ671549	4
PPA	Spain	NK	1984	–	I	KJ526362	4
KK262	Zaire (DRC)	–	1989	1992	I	KJ526364	2
KK202	Zaire (DRC)	–	1974	1974	I	KJ526363	2
K49	Zaire (DRC)	1949	1983	–	I	KJ671543	2
Ndjassi-77	Zaire (DRC)	1977	1979	–	I	KM236553	2
Sylva 1	Angola	1982	1982	–	I	KJ526365	2
Mfuati-79	Congo (People's Republic of Congo)	1979	1980	–	I	KJ526368	2
Congo-73	Zaire (DRC)	NK	1983	–	I	KJ671545	2
M78	Mozambique	NK	1978	–	V	KJ671548	3
MK200	Mozambique	–	1980	1980	V	KJ526369	3
Stavropol 01/08	Russia	2008	2009	–	II	JQ771686	8
TSP 80	Tanzania	NK	1967	–	X	KJ526361	5
TSP80/300	Tanzania	–	1986	1986	X	KJ526366	5
Bartlett	Kenya	NK	1961	–	X§	KJ526356	ND
Uganda	Uganda	NK	1984	–	X	KJ526359	7
Magadi	Kenya	NK	1984	–	X§	KJ526358	ND
Davis	Kenya	NK	1986	–	X§	KJ526357	ND
TS-7	Tanzania	NK	1967	–	X	KJ526360	6
Rhodesia	Rhodesia (Zimbabwe)	NK	1986	–	VIII	KJ671542	8
Spenser	Republic of South Africa	NK	1985	–	?¶	KJ526354	2

*The isolates were selected from the African swine fever virus (ASFV) collection at the National Research Institute for Veterinary Virology and Microbiology (VNIIVViM) in Pokrov, Russia. ASFV genotypes are assigned according to p72 (B646L) nucleotide sequencing and phylogenetic tree reconstruction. Serogroups are defined on the basis of results of hemadsorption inhibition assay (HAI) with reference serum (serogroups 1–8). ASFV isolates from primary outbreaks as well as attenuated variants have been characterized. Democratic Republic of Congo; –, not applicable; NK, not known; ND, not defined.
†If a country name changed since the virus was isolated, the current name of the country is shown in parentheses.
‡Deposited into the collection at VNIIVViM.
§Isolates Bartlett, Magadi, and Davis could not be placed into an existing serogroup because no HAI was observed with available reference serum, so exact serogroups for these isolates are not defined.
¶Isolate Spenser does not belong to any of the 22 known genotypes and remains untyped.

are located in countries where multiple mechanisms of ASF transmission (mixed sylvatic and domestic cycle) are established (*11*).

HAI serology provides a measure of ASFV typing that, compared with p72 genotyping, better discriminates biologically pertinent phenotypes. Viruses belonging to one p72 genotype may be serotypically heterogeneous: strains that are closely related genetically, even from a single isolate, may have different phenotypes and form homologous serogroups. Our assessment of ASFV genotyping relating to HAI serotyping shows the serologic diversity within a p72 genotype. Our results highlight the potential for using serogroup classification to understand issues of homologous cross-protection among ASFV isolates and virus determinants that influence disease emergence.

The key finding from our study is that p72 ASFV phylogenetic analysis fails to accurately define ASFV HAI serogroups. Thus, conventional ASFV genotyping cannot discriminate between viruses of different virulence or predict the efficacy of a specific ASFV vaccine. This finding also suggests that for vaccination-based control of ASF, it may be more important to determine serogroups rather than genotypes of ASFV isolates. Nevertheless, more ASFV sampling is needed to further define genotypes and serogroups.

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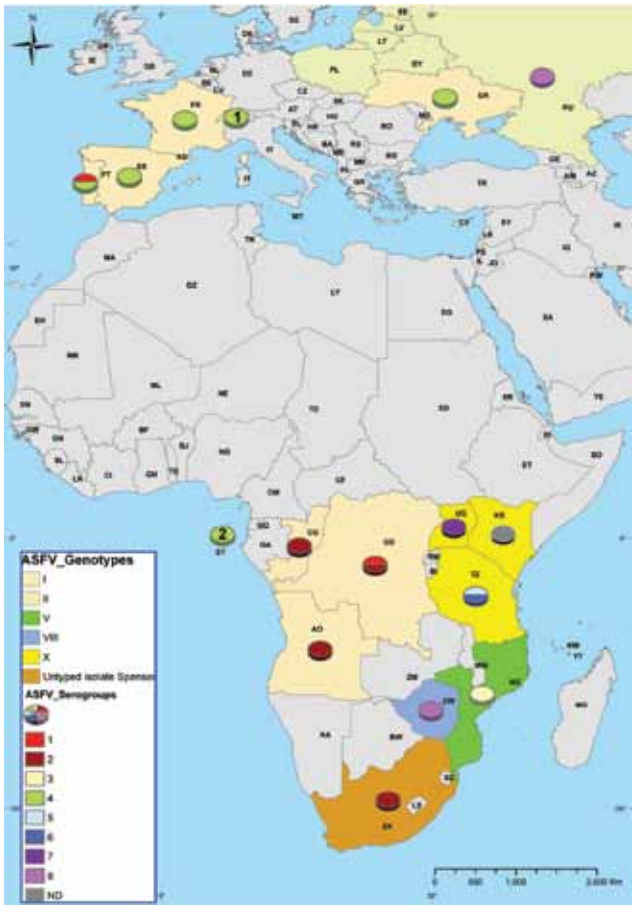

Figure 2. World distribution of African swine fever virus (ASFV) isolates maintained in a collection at the National Research Institute for Veterinary Virology and Microbiology in Pokrov, Russia. Results of p72 genotyping and hemadsorption inhibition assay of ASFV isolates are summarized on the map. Genotype II of ASFV isolates from Lithuania, Latvia, Estonia, Poland, and Belarus was identified by CISA-INIA (Animal Health Research Center; European Union Reference Laboratory for African Swine Fever). ASFV isolate O-77, which was isolated in 1977 from Odessa, Ukraine (at the time, part of the Union of Soviet Socialist Republics), was used in this study. On the basis of CISA-INIA results, currently circulating isolates in Ukraine belong to genotype II. ASFV isolate Brazil-80 (genotype I, serogroup 4) is not shown. The oval with a 1 inside indicates Switzerland; the oval with a 2 inside indicates São Tome and Principe. Country names are presented as 2-letter country codes as designated by the International Organization for Standardization country codes (ISO 3166, http://www.iso.org/iso/country_codes.htm).

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Murine Typhus, Reunion, France, 2011–2013

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Murine typhus case was initially identified in Reunion, France, in 2012 in a tourist. Our investigation confirmed 8 autochthonous cases that occurred during January 2011–January 2013 in Reunion. Murine typhus should be considered in local patients and in travelers returning from Reunion who have fevers of unknown origin.

Murine typhus, an acute zoonotic infection caused by *Rickettsia typhi* (*1*), occurs worldwide. It is underdiagnosed and largely underreported because of its nonspecific characteristics and frequently mild course, a lack of active monitoring; and limited awareness among physicians (*2*). Symptoms include fever, headache, and inconsistent and transient rashes (*1*,*3*). Serious complications have been associated with acute infections (*4*). The death rate is generally low but can reach 4% without the use of antibacterial drugs (*1*,*4*). The classic cycle involves rats (black rat [*Rattus rattus*] and brown rat [*R. norvegicus*]) in urban areas and the rat flea *Xenopsylla cheopis* (*3*). However, murine typhus is also documented in suburban areas, where opossums, cats, dogs, and their fleas coexist (*3*).

Reunion is a French overseas territory of 2,512 km^2 in the southwestern Indian Ocean, 700 km east of Madagascar, and has a tropical climate. Most of its 850,000 residents live in coastal areas where major towns are located. Invasive rodents (rats, including the black rat, mice, and shrews) and endemic rodents (i.e., the tailless tenrec [*Tenrec ecaudatus*]) are present both in rural and urban settings (*5*). *X. cheopis* and *X. brasiliensis* fleas are present mainly on rats, but their presence and their abundance vary according to the local climates of the island and to the seasons (*6*). Stray dogs and feral cats are numerous in the island and often are infested with fleas. In addition, domestic dogs and cats traditionally are allowed to run freely in both urban and rural areas.

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In early 2012, murine typhus was reported in a tourist returning from Reunion (*7*). We conducted a study to collect epidemiologic data and to describe clinical manifestations for all murine typhus cases diagnosed in the infectious disease departments of hospitals in Reunion.

The Study

In agreement with infectious disease practitioners and the World Health Organization (WHO) Collaborating Centre for Rickettsial Diseases, we chose to research murine typhus only in cases of fever of unknown origin lasting >7 days. In accordance with WHO Collaborating Centre procedures, a case was considered confirmed by seroconversion or by a ≥4-fold increase in antibody response against *R. typhi* from acute- and convalescent-phase serum samples; by a positive indirect immunofluorescence test for typhus-group rickettsiae confirmed by Western blot; or by positive *R. typhi*–specific real-time quantitative PCR. We used Western blot to formally distinguish murine typhus from epidemic typhus. Routine analysis (i.e., blood cell count) was performed in Reunion. Murine typhus diagnostic tests were performed by the WHO Collaborating Centre for Rickettsial Diseases (Marseille, France) (*7*).

We collected and analyzed data for each case by reviewing clinical records and administering an epidemiologic questionnaire by phone to each patient. The following data were collected: symptoms, signs, and complications during illness; results of routine laboratory analyses; treatment and outcome; habitat and environmental characteristics; presence of pets, livestock, or rodents; contact with fleas; daily activities in the 15 days before symptom onset; and travel during the previous 3 months.

During January 2011–January 2013, a total of 8 autochthonous murine typhus cases were confirmed: 5 patients in 2012, 1 in 2013, and 2 who tested positive for retrospective screening of archived serum samples from 2011 (Figures 1, 2; Table). Seven cases occurred during the Southern Hemisphere summer. All the patients lived in private houses in the western and southern parts of the island in periurban areas. Patients' average age was 46 years (range 21–66 years), and the male:female ratio was 1:1.

For all patients, fever lasted an average of 14.3 days (range 10–21 days). Six patients were hospitalized. The 2 other main symptoms were arthromyalgia (7 patients) and headaches (6 patients). Only 4 patients had a maculopapular

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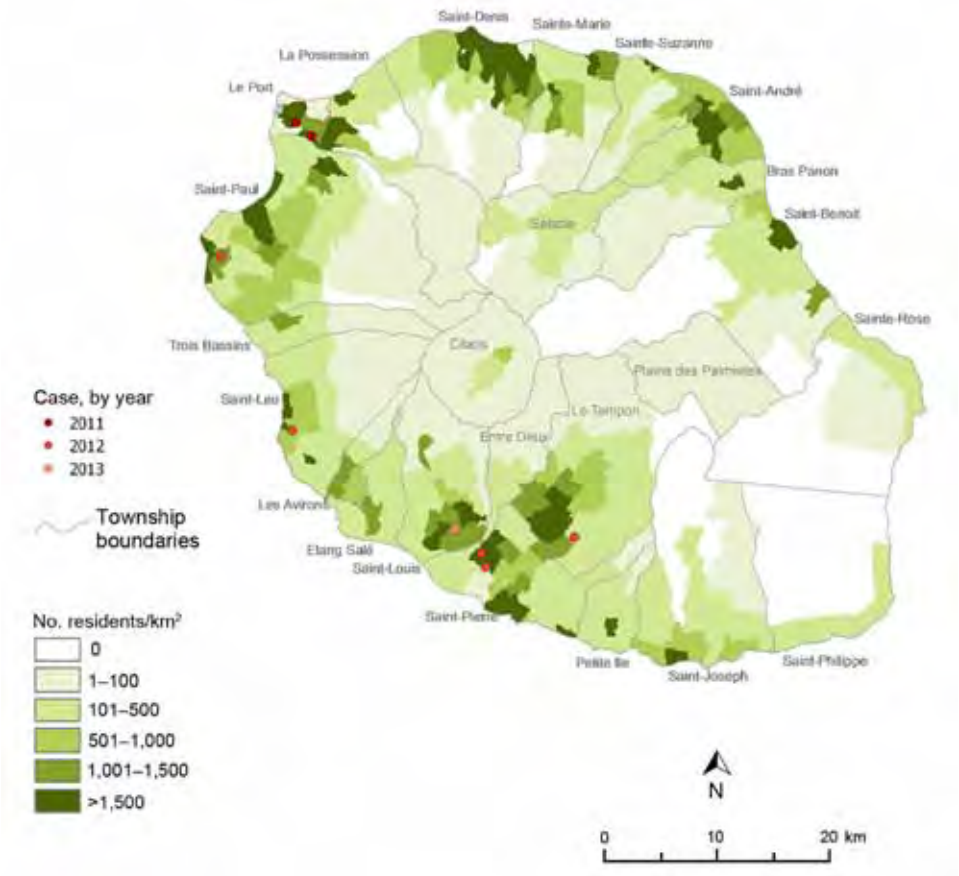

Figure 1. Locations of murine typhus cases, Reunion, France, January 2011–January 2013.

rash, but no inoculation eschar was found. In addition, pharyngitis developed in 4 patients. In contrast, gastrointestinal symptoms and ophthalmologic signs, such as uveitis, developed in 2 patients each. One patient had confusion, and 1 had prostration.

Six patients had elevated liver enzymes (reference values aspartate aminotransferase and alanine aminotransferase >33 UI/L; lactate dehydrogenase >480 UI/L). Early lymphopenia (lymphocytes <1,000 cells/mL) occurred in 5 patients, and thrombocytopenia (platelets <150 000/mL) in 4. Renal dysfunction did not develop in any of the 8 patients.

Six patients were treated with doxycycline (2 patients in the acute phase for whom fever decreased during the following 24 hours; 4 others despite previous spontaneous resolution of fever). One of the 2 remaining patients, a pregnant woman, was treated with spiramycin without any complications to the baby during 6 months of follow-up. The other patient did not receive any treatment. All patients recovered completely, although they had weakness lasting at least 1 month.

None of the patients had traveled overseas during the previous 3 months, and no other cases had been identified nearby. Only 1 patient reported having recent insect bites and pruritus. No patient recalled contact with rats in his/her living environment before disease onset.

Five patients completed the investigation questionnaire. Reported risk factors included close contact with domestic pets (4 patients); presence of livestock in the surroundings (4 patients); presence of wild fauna (rats, tenrecs, dogs, cats) in the surroundings (4 patients); rat

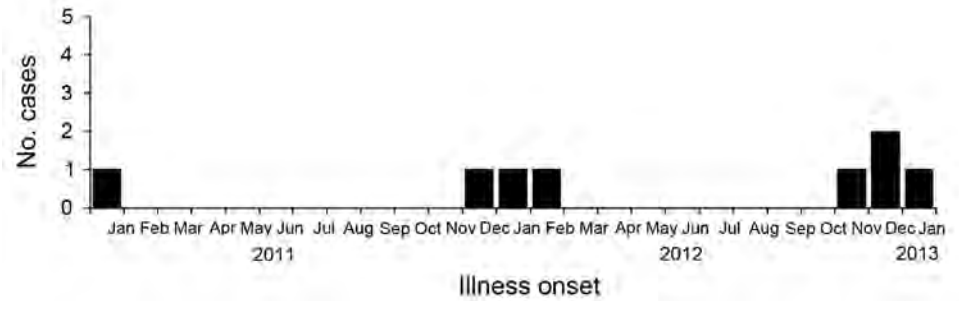

Figure 2. Temporal distribution of murine typhus cases, Reunion, France, January 2011–January 2013.

Table. Confirmation of 8 murine typhus cases, Reunion, France, January 2011–January 2013*

Pt no.	Age, y/sex	Onset date	Onset to first sample, d	First test result		First to second sample, d	Second test result		Second to third sample, d	Third test result		Confirmatory test results		Onset to PCR, d
				IgM†	IgG‡		IgM†	IgG‡		IgM†	IgG‡	WB	PCR	
1	55/M	2011 Jan	18	512	256	76	0	256				*R. typhi*	ND	
2	66/M	2011 Dec	30	512	128	63	128	128				*R. typhi*	ND	
3	41/F	2012 Jan	33	512	128	66	512	256				*R. typhi*	Neg	17
4	49/M	2012 Feb	14	0	0	22	512	128	39	128	128	*R. typhi*	Neg	14
5	47/F	2012 Nov	12	256	128	26	256	512				ND	ND	
6	50/M	2012 Dec	11	0	0	19	512	256				ND	Pos	11
7	21/F	2012 Dec	9	128	128	48	512	256				ND	ND	
8	39/F	2013 Jan	37	512	128	172	0	512				ND	ND	

*All samples were serum samples. Blank cells indicate a third serum sample was not tested. ND, not done; neg, negative; pos, positive; pt, patient; WB, Western blot.
†IgM pos if titer >1:64.
‡IgG pos if titer >1:128.

extermination (3 patients); outdoor activities, such as jogging, picnicking, walking, gardening (3 patients); or house cleaning (2 patients).

Conclusions

Serologic and molecular evidence is sufficient to indicate that autochthonous transmission of murine typhus exists in Reunion. The real impact and the origin (always present but only recently identified vs. recent importation by navigation trade from Asia or from Madagascar) of murine typhus remain unknown. Murine typhus is common in tropical areas but is often not distinguished from other more prevalent febrile bacterial infections, such as scrub typhus, spotted fever rickettsioses, and leptospirosis. However, murine typhus recently has been shown to be a major cause of fever of unknown origin in Indonesia and Laos (*8*,*9*). In Reunion, despite a systematic biologic investigation (dengue, chikungunya, and leptospirosis) of dengue-like syndrome, many fevers still remain of unknown origin (*10*,*11*). Our results support the hypothesis that murine typhus could be a major cause of fever in Reunion.

Like leptospirosis, murine typhus seems to peak seasonally during the summer (*12*). As previously described in the tropics, climatic conditions during the hot and wet seasons lead to the proliferation of rats and so increase human exposure to murine typhus (*4*,*13*). Because most of the 8 patients had contact with pets, the role of fleas that infest pets (e.g., *Ctenocephalides felis*) in the transmission of murine typhus needs to be clarified, as does the role of *X. cheopis*, the rat flea (*14*).

In conclusion, murine typhus is an emerging disease in Reunion. It is possibly underdiagnosed and has the potential to cause major illness (*9*). This disease should be considered locally when fevers of unknown origin are investigated, as well as in travelers with fever returning from the island. Specific studies are needed to assess the effect of murine typhus on public health and to describe the epidemiology of the disease. The best way to prevent murine typhus is to minimize exposure to ectoparasite vectors by limiting contact with rodents and fleas. Such measures include keeping a well-maintained yard, minimizing waste, protecting pets from fleas, and using mechanical or chemical protection in case of at-risk activities.

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Awareness and Support of Release of Genetically Modified "Sterile" Mosquitoes, Key West, Florida, USA

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After a dengue outbreak in Key West, Florida, during 2009–2010, authorities, considered conducting the first US release of male *Aedes aegypti* mosquitoes genetically modified to prevent reproduction. Despite outreach and media attention, only half of the community was aware of the proposal; half of those were supportive. Novel public health strategies require community engagement.

Two rapidly emerging viruses, chikungunya and dengue, are spread by *Aedes aegypti* mosquitoes (*1*). Vector population control strategies have had variable success, and control by using genetically modified (GM) mosquitoes is under consideration (*2*). In trials, 1 GM variant, the OX513A *Ae. aegypti*, has survived under field conditions and reduced wild-type populations (*3*,*4*). However, there were concerns among public health officials, ecologists, and entomologists that the measures used to engage and inform local communities were too limited (*5*,*6*). Community support has been linked to the success (*7*) and failure (*8*) of vector and pest control campaigns.

The Study

During 2009–2010, an outbreak of dengue fever occurred in Key West, Florida (*9*). Shortly thereafter, the Florida Keys Mosquito Control District proposed the first release of a GM mosquito, OX513A *Ae. aegypti*, in the United States. The proposal was met with controversy.

On publication of this article, the release was undergoing inspection by the US Food and Drug Administration and had not occurred.

We conducted a survey in June 2012 to examine awareness and support of the release after 80 media and outreach activities had been conducted in Key West and Stock Island, Florida. We randomly selected 400 residences from the Monroe County Property Appraisers Office database and administered a cross-sectional knowledge, attitudes, and practices survey about mosquito control and dengue virus.

We collected information on demographics, perception of dengue risk, mosquito knowledge and prevention activities, and health care–seeking behavior, among other topics. Support was determined on a scale of 1 (strongly oppose) to 5 (strongly support). We requested reasons for participants' level of support; themes raised by ≥9 respondents were coded into study categories by 2 investigators (K.C.E. and M.H.H.).

In this study, the use of GM male mosquitoes results in death of offspring in the larval or pupal stage of gestation; because of this outcome, outreach activities in the area preceding the survey referred to the mosquitoes as "sterile." The survey we used included "sterile" because this term had been used in community awareness activities and should have been familiar to those who had heard of the proposed release, and we added "genetically modified" as a descriptor of the mosquitoes.

We divided participant groups into participants into those who had or had not heard of the release plans. We used logistic regression to assess associations between hearing of the release and possible explanatory factors. Missing values for household income were imputed. Distribution of levels of support of the release among those who had heard of the plan was stratified and tested for differences by demographic factors and participation in dengue and mosquito awareness and prevention activities. We used the Mantel-Haenszel test for trend for ordinal variables (e.g., education, income) and the χ^2 test of heterogeneity for categorical variables. We used ANOVA, a nested analysis of variance approach, for continuous variables.

Of the 400 participants (Table 1), 75 (18.8%) were from the originally selected households. Of the 386 participants who responded to the question of whether they had heard of the proposed release before the survey, 195 (51.1%) answered "yes." Prior awareness was more common in white non-Hispanics, residents with income levels >$50,000 per year, older adults, those who resided on Key West Island, and residents with knowledge of the local Action to Break the Cycle of Dengue public health campaign (Table 1). Among the 195 who were aware of the release, the distribution of support was: 9.7% strongly

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Table 1. Comparison of 400 surveyed local residents who had heard of release of genetically modified "sterile" male *Aedes aegypti* OX513A mosquitoes to those who had not, Key West, Florida, USA*

Response	Sample distribution, no. (%)†	Heard, %‡	Not heard, %	Imputed unadjusted OR (95% CI), p value	Average adjusted OR (95% CI), multiply imputed data
Age, y					
18–35	98 (25.1)	19	81.1	1 (Referent)	1 (Referent)
36–50	77 (19.7)	55.3	44.7	5.16 (2.61–10.2), <0.001	3.75 (1.75–8.03), <0.001
51–65	121 (31.0)	71.8	28.2	10.5 (5.48–20.1), <0.001	8.17 (3.95–16.9), <0.001
≥66	94 (24.1)	56.8	43.2	5.51 (2.84–10.7), <0.001	6.80 (3.14–14.7), <0.001
Sex					
M	214 (53.9)	56	44	1 (Referent)	1 (Referent)
F	183 (46.1)	45.3	54.8	0.65 (0.43–0.97), 0.03	0.58 (0.35–0.95), 0.03
Region of Key West					
Old Town	153 (38.4)	51	49	1 (Referent)	1 (Referent)
Midtown	61 (15.3)	55.2	44.8	1.17 (0.64–2.16), 0.60	1.33 (0.62–2.85), 0.46
New Town	126 (31.6)	57.4	42.6	1.29 (0.80–2.09), 0.29	1.53 (0.82–2.83), 0.18
Stock Island	59 (14.8)	33.9	66.1	0.49 (0.26–0.93), 0.03	0.65 (0.29–1.44), 0.29
Race/ethnicity					
White non-Hispanic	247 (66.9)	63	37	1 (Referent)	1 (Referent)
White Hispanic	46 (12.5)	36.4	63.6	0.33 (0.17–0.64), 0.001	0.47 (0.21–1.04), 0.06
Black	38 (10.3)	30.3	69.7	0.24 (0.11–0.53), <0.001	0.36 (0.15–0.90), 0.03
Other	38 (10.3)	27	73	0.21 (0.10–0.42), <0.001	0.25 (0.11–0.56), <0.001
Household income					
<$35,000	54 (13.5)	44	46	0.29 (0.14–0.60), <0.001	0.75 (0.31–1.82), 0.53
$35,000-$49,999	31 (7.8)	41.9	48.1	0.37 (0.15–0.90), 0.03	0.83 (0.30–2.30), 0.72
$50,000-$74,999	52 (13.0)	64.7	35.3	0.63 (0.30–1.32), 0.22	0.92 (0.41–2.08), 0.85
$75,000-$99,999	37 (9.3)	54.1	46	0.50 (0.22–1.15), 0.10	0.77 (0.31–1.92), 0.58
≥$100,000	72 (18.0)	70.8	29.2	1 (Referent)	1 (Referent)
Education level					
High school or lower	123 (31.6)	34.8	65.2	1 (Referent)	1 (Referent)
Some college	77 (19.8)	45.3	54.7	1.54 (0.85–2.79), 0.15	1.71 (0.83–3.54), 0.15
Associate's degree	19 (4.9)	68.4	31.6	3.83 (1.35–10.8), 0.01	5.73 (1.61–20.3), 0.007
Bachelor's degree	107 (27.5)	55.7	44.3	2.38 (1.38–4.08), 0.002	1.93 (0.97–3.81), 0.059
Graduate or professional degree	63 (16.2)	77.8	22.2	6.63 (3.27–13.4), <0.001	3.37 (1.42–8.02), 0.006
Aware of ABCD§					
No	48 (19.0)	59.6	40.4	1 (Referent)	1 (Referent)
Yes	252 (81.0)	79.2	20.8	2.56 (1.27– 5.14), 0.008	2.32 (1.04–5.17), 0.04

*All variables listed are included in the adjusted model.
†Demographic totals may not add up to 400 because some participants refused to report demographic information.
‡Percentages reflect within category percentages.
§ABCD, Florida Keys-based Action to Break the Cycle of Dengue public health campaign.

opposed, 8.2% opposed, 25.1% neutral, 22.1% supportive, and 34.9% strongly supportive. Men, less educated persons, and those willing to pay $100 or more for mosquito control were more likely to be strongly supportive (Table 2). The most common reasons for opposing the release were disturbance of nature and that it was an unproven technology. Most supporters of the release expressed a desire to do anything to get rid of mosquitoes or preferred the method to chemicals and spraying (Figure). On the basis of effectiveness, safety, and/or lack of unintended consequences, 22 of the 195 indicated that their support was conditional.

Conclusions

For community acceptance of the release of GM mosquitoes, several issues must be addressed. Release of GM mosquitoes into the community should be transparent; therefore, the Florida Keys Mosquito Control District has begun to disseminate information through public events, articles, and presentations. Identification of solutions to reduce risk for vector-borne disease should involve stakeholders from the public, and community leaders in public health, vector control, and municipal administrators. Open communication with community members and stakeholders through a health advisory board was instrumental in quelling a 1989 invasion of Mediterranean fruit flies in California that had become a crisis event (*10*). In Key West and Stock Island, public awareness and communication campaigns had limited success. Awareness of the release varied across sections of the city and by demographic group. At the time of the survey, the release was planned for Key West; in Stock Island, awareness was much lower. Adjacent areas should be included in communications because residents and *Ae. aegypti* are mobile. (*11*). Knowledge of current events has been associated with gender, education level, race and ethnicity, and age (*12*). Outreach should target groups with a tendency towards lower awareness of public health measures.

Table 2. Percentage of responses to demographic, dengue and mosquito-related factors according to level of support for a release of genetically modified "sterile" mosquitoes in Key West, Florida, USA, among the 195 participants who had heard of the release*

Response	Strongly opposed	Somewhat opposed	Neutral	Somewhat supportive	Strongly supportive	p value
Overall level of support, no. (%)	19 (9.7)	16 (8.2)	49 (25.1)	43 (22.1)	68 (34.9)	NA
Mosquitoes noticed outside (%, many or very many)	26.3	12.5	14.6	11.9	22.1	0.87†
How many days did you spend outside last week, % ≥3 d	79.0	68.8	83.7	79.1	75.0	0.75†
Limit outdoor activity because of mosquitoes, % often or always	10.5	6.3	10.2	4.7	8.8	0.80†
Able to report dengue as a mosquito-carried disease, % yes	84.2	87.5	75.5	79.1	80.9	0.78†
How serious is dengue in Key West, % very or extremely serious	31.6	43.8	38.6	40.0	31.3	0.63†
How likely is it that you or a family member will get dengue in Key West, % somewhat or very likely	10.5	18.8	10.6	12.8	10.8	0.78†
Aware of ABCD, % yes	15.8	18.8	26.1	29.0	14.3	0.68†
Willing to pay $100 or more for effective mosquito control, %, yes	28.6	50.0	58.7	73.5	73.3	<0.001†
Current mosquito control is very or extremely effective, % yes	66.7	75.0	75.5	69.8	72.1	0.97†
Mean age, y	57.8	52.6	54.7	56.2	57.7	0.67‡
Distribution of support by category						
Sex						<0.001§
M	10.5	6.1	18.4	17.5	47.4	ND
F	8.6	11.1	34.6	28.4	17.3	ND
Key West region						0.29§
Old Town	13.3	8.0	25.3	20.0	33.3	ND
Midtown	3.1	9.4	34.4	25.0	28.1	ND
New Town	7.3	7.3	26.1	17.4	42.0	ND
Stock Island	15.8	10.5	5.3	42.1	26.3	ND
Race						0.21§
White non-Hispanic	9.2	8.5	24.2	20.9	37.3	ND
White Hispanic	6.3	0.0	18.8	37.5	37.5	ND
Black	0.0	10.0	50.0	20.0	20.0	ND
Other race	30.0	20.0	10.0	20.0	20.0	ND
Household income						0.16†
<$35,000	13.6	13.6	18.2	27.3	27.3	ND
$35,000-$49,999	7.7	15.4	7.7	46.2	23.1	ND
$50,000-$74,999	12.5	3.1	21.9	18.8	43.8	ND
$75,000-$99,999	10.0	10.0	30.0	25.0	25.0	ND
≥$100,000	3.9	7.8	27.5	13.7	47.1	ND
Highest level of education						0.09†
Lower than high school	0.0	0.0	25.0	25.0	50.0	ND
High school graduate	0.0	5.6	19.4	25.0	50.0	ND
Some college	15.6	12.5	25.0	25.0	21.9	ND
Associate degree	7.7	0.0	30.8	23.1	38.5	ND
Bachelor's degree	11.9	10.2	28.8	18.6	30.5	ND
Graduate or professional degree	12.2	6.1	24.5	20.4	36.7	ND

*NA, not applicable; ND, calculation not done; ABCD, Florida Keys-based Action to Break the Cycle of Dengue public health campaign.
†p value for trend calculated by using Mantel-Haenszel test.
‡p value calculated by using nested analysis of variance.
§p value calculated by using χ^2 test for heterogeneity.

Support was more commonly reported than opposition among those aware of the release; a large portion was neutral. Most neutral respondents reported they did not know enough to make a decision, and many supporters wanted more information or had concerns. To progress from awareness to knowledge, to understanding, and then to decision-making would require considerable effort and improvement in overall scientific literacy (*13*). The scientific community is divided about the amount of information that should be provided to community members on highly technical vector control strategies such as the release of the OX513A mosquito (*14*). Benchmarks for acceptable engagement and support should be set by public health organizations before GM vector releases are planned, which will require input from scientists, stakeholders, and the community.

Strongly opposed participants most commonly reported unintended consequences or disturbing natural ecosystems as their reason for opposition. Conversely, some supporters considered the mosquito release a more natural way of controlling mosquito populations than insecticides. This was substantiated in a follow-up study (*15*).

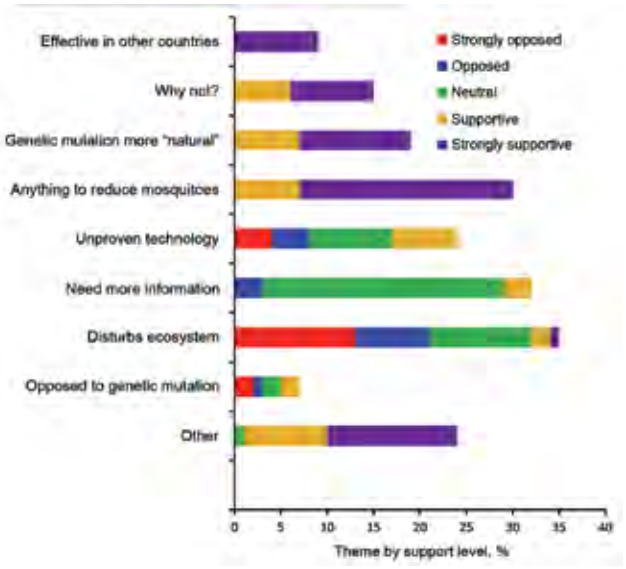

Figure. Proportion of respondents reporting different themes for their level of support of plans to release genetically modified, referred to as "sterile," male mosquitoes on Key West, Florida, USA.

This study has several limitations. Participants may not have fully represented the community because of seasonal housing closures and inaccessibility of some gated communities. A systematic replacement strategy was used to minimize bias. To obtain information on support, we provided a short statement about the release, modeled after earlier community outreach efforts and that used the term "sterile mosquito" instead of "genetically modified mosquito." We excluded responses of participants without prior awareness from our analysis because our informational statement was cursory. Follow-up studies in Key West that provided more extensive information yielded the same 9% strong opposition rate (*15*).

Introduction of GM mosquitoes has the potential to reduce mosquito-borne disease; however, little data exist on the type and extent of outreach required or community support needed to reduce opposition. As of December 2014, a short-term release of Oxitec OX513A mosquitoes is proposed on Key Haven, a peninsula adjacent to Key West. This is part of an application by Oxitec: Regulatory Clearance for Investigational Use of a New Animal Drug. This release is proposed before broader implementation in Key West or elsewhere in the Florida Keys (M.S. Doyle, unpub. data). If approved, this release could serve as a model of best practices for establishing community relations and engagement before implementing vector control strategies.

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A disclosure statement was read to each participant. The protocol was approved by the University of Arizona Human Subjects Research Committee and deemed exempt.

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Novel *Candidatus* Rickettsia Species Detected in Nostril Tick from Human, Gabon, 2014

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We report the identification of a nymphal nostril tick (*Amblyomma* sp.) from a national park visitor in Gabon and subsequent molecular detection and characterization of tickborne bacteria. Our findings provide evidence of a potentially new *Rickettsia* sp. circulating in Africa and indicate that tick bites may pose a risk to persons visiting parks in the region.

Ticks are hematophagous arthropods that parasitize different species of vertebrates, and they serve as intermediate hosts for infectious pathogens that can have serious implications for humans. Because of climate change and socioeconomic factors, tickborne diseases have increased in the past 3 decades, and these arthropods are second only to mosquitoes as vectors of human infectious diseases (*1*,*2*). Many ixodid tick species are found in Africa, and tickborne diseases in travelers returning from that continent have been reported worldwide (*3*). Among the travel-associated cases of African tick-bite fever, most occur in persons returning from travel to southern Africa with fever and systemic illness (*4*).

The Study

A 21-year-old female field worker from Spain visited Lopé National Park in Gabon (Africa) for 13 days during January–February 2014 to observe chimpanzees and gorillas. Four days before returning to Spain, she noticed a foreign body (black spot) inside her left nostril but had no signs or symptoms of illness. After returning home, the woman sought care at the Tropical Medicine Centre at University Hospital Ramón y Cajal in Madrid, Spain, where a tick attached to the anteroinferior part of the left nasal septum (the Kiesselbach area) was extracted with forceps during rhinofibroscopy. The tick was sent to the Center of Rickettsiosis and Arthropod-Borne Diseases at Hospital San Pedro–Center of Biomedical Research of La Rioja in Logroño, Spain, for identification and molecular detection of tickborne bacteria. The tick was photographed (Figure) and identified, by morphologic features, as an *Amblyomma* sp. nymph, according to taxonomic keys (*5*).

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Immature stages of *Amblyomma* ticks cannot be identified to the species level on the basis of morphologic features without allowing the nymph to molt. Thus, we conducted genetic analysis to identify the tick. To extract genomic DNA, we incubated the tick with ammonium hydroxide (1 mL of 25% ammonia and 19 mL of sterile water) for 20 min at 100°C and for another 20 min at 90°C. The DNA was used as template in PCR assays targeting the tick mitochondrial 16S rRNA (*6*), mitochondrial 12S rRNA (*7*), and nuclear 5.8S-28S rRNA intergenic transcribed spacer 2 (ITS2) (*8*). As a positive control, we used DNA extract from a tick of known identity (*Haemaphysalis punctata*) that was collected in La Rioja, Spain.

Subsequent detection and molecular characterization of tickborne bacteria (*Rickettsia* spp., *Anaplasma phagocytophilum*, and *Borrelia* spp.) were performed. To screen for the presence of rickettsiae, we used PCR assays targeting 2 fragments of the *ompB* gene (511 bp and 811 bp, respectively) (*9*,*10*). Four additional genetic markers were used to classify the isolated rickettsia to the species level: fragments of *ompA* (532 bp) (*11*,*12*), 16S rRNA (1,500 bp) (*13*), *sca4* (623 bp) (*14*), and *gltA* (1,019 bp) (*15*). These markers were amplified in accordance with the taxonomic scheme for classifying rickettsiae at the genus and species levels (online Technical Appendix, reference *16*, http://wwwnc.cdc.gov/EID/article/21/2/14-1048-Techapp1.pdf).

To screen for the presence of *A. phagocytophilum* and *Borrelia* spp., we performed PCR targeting the partial *msp2* gene (334 bp) and *Borrelia* genus–specific 16S rRNA (1,350 bp) gene (online Technical Appendix, references *17*,*18*). Each PCR included a positive control: *Rickettsia slovaca* strain S14ab DNA (obtained from Vero cells that had been inoculated in our facility with homogenate of an *R. slovaca*–infected *Dermacentor marginatus* tick from La Rioja Province); *A. phagocytophilum* strain Webster DNA (provided by D. Raoult [Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Marseille, France] and J.S. Dumler [Johns Hopkins Hospital, Baltimore, MD, USA]); or *Borrelia burgdorferi* sensu stricto DNA (provided by V. Fingerle [German National Reference Centre for *Borrelia*, Oberschleissheim, Germany]). DNA-free water was included as a negative control in each set of reactions.

We used BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) to compare sequences generated by each pair of

Figure. *Amblyomma* sp. nymphal tick removed from the nostril of a woman who visited Lopé National Park in Gabon (Africa), 2014. Scale bar represents 1 mm.

primers with sequences in GenBank. The 16S rRNA sequence showed highest identity (91% [368/405 bp]) with the mitochondrion *Amblyomma variegatum* 16S rRNA gene (GenBank accession no. L34312). The 12S rRNA and ITS2 sequences reached only 89% and 91% identity, respectively, with those of *A. variegatum* and were closest (94.3% [298/316 bp] and 99% identity [809/817 bp], respectively) to those for an *Amblyomma* sp. nymph (GenBank accession nos. KC538944 and KC538941). Of interest, the 12S rRNA and ITS2 sequences also corresponded to those of a nostril tick removed from a researcher who had been visiting a national park in Uganda (online Technical Appendix, reference *19*); the 16S rRNA sequence for the tick from this researcher was not in GenBank. The levels of sequence similarities that we found did not enable species determination of the tick in this study. Two previous reports about nostril ticks in humans who have visited Africa are available (online Technical Appendix, references *20*,*21*).

For the strain in this study, single bands of the expected sizes for the 2 *ompB* rickettsial fragment genes analyzed were detected. A BLAST search revealed that these 2 sequences were genetically most similar (97.2% and 98.3% identity) to the *ompB* gene of *Rickettsia japonica* and *Rickettsia heilongjiangensis*, respectively (Table). The nucleotide sequence of *ompA* was closest (99.8% identity) to that of the *ompA* of *Rickettsia* sp. strain Davousti, and showed maximum identity (97.2%) with *R. heilogjiangensis* as validated species. When compared with sequences of validly published *Rickettsia* spp. available in GenBank, the 16S rRNA and *sca4* gene sequences showed the highest identity with *R. japonica* (99.4%–99.6% and 98.5%, respectively). The *gltA* sequence shared 99.1% identity with *R. japonica* and *R. heilogjiangensis* (Table). These results are in accordance with the genetic criteria for identifying the rickettsia as *Candidatus* Rickettsia sp. (online Technical Appendix, reference *16*).

Although reports about the circulation of *Rickettsia* spp. in Gabon are scarce, *Rickettsia* sp. strain Davousti was detected in *Amblyomma tholloni* ticks from African elephants in that country (online Technical Appendix, reference *22*). In addition, the *gltA* sequence obtained in our study was 99.9% identical to the *gltA* sequence of *Rickettsia* sp. strain Davousti (Table). These findings suggest that both strains could belong to the same *Rickettsia* sp. No other sequences, apart from those for *ompA* and *gltA*, of *Rickettsia* sp. strain Davousti have been deposited in GenBank; however, on the basis of findings in the previous report (online Technical Appendix, reference *22*), we propose the name *Candidatus* R. davousti for the strain in this study.

In addition, we did not obtain amplicons for the *msp2* gene of *A. phagocytophilum* or 16S rRNA gene specific for *Borrelia* genus. For each set of PCR primers, no bands were detected on agarose gels for negative control samples.

The partial tick mitochondrial 16S rDNA, 12S rDNA, and ITS2 sequences have been deposited in GenBank under accession nos. KJ619630, KJ619636, and KJ619637, respectively. The partial *ompB* (2 fragment genes), *ompA*, 16S rRNA, *sca4*, and *gltA* sequences of the novel tick-derived rickettsia in this study have been deposited in GenBank under accession numbers KJ619632, KJ619633, KJ619631, KJ619629, KJ619634, and KJ619635.

Conclusions

We report the detection of a potentially novel *Rickettsia* sp. from an *Amblyomma* sp. nymphal tick that was removed from the nostril of a field researcher when she returned to Spain after visiting Gabon's Lopé National Park; we propose the name *Ca*. R. davousti for this *Rickettsia* sp. strain.

Table. Maximum identities of rickettsial sequences obtained from an *Amblyomma* sp. tick from Gabon with validated *Rickettsia* spp. published in GenBank*

Gene sequence, GenBank accession no.	% identity with *Rickettsia* spp. (basepairs)†		
	R. japonica, GenBank accession no. AP011533	*R. heilongjiangensis*, GenBank accession no. CP002912	*Rickettsia* sp. Davousti, GenBank accession nos. DQ402516–402517
ompB, KJ619633	97.2 (442/455)	96.9 (441/455)	NA
ompB, KJ619632	96.9 (746/770)	98.3 (742/755)	NA
ompA, KJ619631	96.1 (472/491)	97.2 (477/491)	99.8 (490/491)
16S rRNA, KJ619629	99.4–99.6 (1369–1373/1378)	99.3–99.6 (1368–1372/1378)	NA
sca4, KJ619634	98.5 (509/517)	98.3 (508/517)	NA
gltA, KJ619635	99.1 (903/911)	99.1 (903/911)	99.9 (691/692)

*The tick was removed in 2014 from the nostril of a woman who returned home to Spain after visiting Lopé National Park in Gabon. NA, not available.
†Percentages of identity with sequences of *Rickettsia* sp. Davousti have been also included when available due to the high level of similarity with our sequences.

Our findings provide further evidence of the presence of circulating *Rickettsia* sp. in Africa and indicate that tick bites may be a threat to persons visiting national parks in Africa. Further studies are needed to determine the prevalence of *Ca*. R. davousti and to establish whether this bacterium is pathogenic for humans.

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Outbreak of Henipavirus Infection, Philippines, 2014

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During 2014, henipavirus infection caused severe illness among humans and horses in southern Philippines; fatality rates among humans were high. Horse-to-human and human-to-human transmission occurred. The most likely source of horse infection was fruit bats. Ongoing surveillance is needed for rapid diagnosis, risk factor investigation, control measure implementation, and further virus characterization.

Henipaviruses belong to a genus of recently emerging viruses within the family *Paramyxoviridae* (*1*–*3*) and include 2 zoonotic members: Hendra virus (HeV) and Nipah virus (NiV). HeV was first described in Australia in 1994, when it caused an outbreak of severe acute respiratory diseases that led to a high mortality rate among horses. Subsequently, several sporadic cases of HeV infection have occurred in horses in Australia; transmission to humans has occurred and the fatality rate was high (*4*,*5*). NiV was first recognized as a human pathogen in peninsular Malaysia in 1998. This outbreak among pig farmers and abattoir workers exposed to infected swine secretions (*6*) was associated with severe encephalitic illness and a high fatality rate. Subsequently, NiV emerged as a major public health problem in Bangladesh and India (*7*–*9*).

The natural reservoir of both viruses is pteropid bats, which harbor the viruses but do not show clinical illness (*3*). Virus transmission from bats to domestic animals is thought to be through pasture or feed contaminated by bat urine, feces, or other excretions (*10*). Transmission of HeV to humans has been invariably associated with close contact with ill horses (*4*), and transmission of NiV in Bangladesh is mainly through date palm sap contaminated with bat secretions (*11*). Human-to-human transmission of NiV also occurs (*12*,*13*).

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The Study

On April 2, 2014, the Philippine National Epidemiology Center received a report of human deaths in 2 villages, Tinalon and Midtungok, in the municipality of Senator Ninoy Aquino, province of Sultan Kudarat, island of Mindanao. The villages are ≈15 km apart, and the provincial referral hospital is in Isulan, 80 km away. An outbreak investigation led by the National Epidemiology Center identified additional human deaths and nonfatal infections and concurrent neurologic disease and sudden deaths in several horses, all of which were subsequently consumed by villagers. On May 12, 2014, the Philippine government asked the World Health Organization for further outbreak investigation assistance.

During May 22–24, 2014, a combined team from the Philippine Department of Health, Department of Agriculture, and the World Health Organization interviewed persons who survived, those with suspected cases, and family members of the deceased and conducted focus group interviews with other persons in affected villages. Key informants from local human and animal health agencies were also interviewed, and hospital records for persons with suspected cases were reviewed. We defined a human case as illness in any person with an epidemiologic link to the municipality of Senator Ninoy Aquino and who had experienced acute encephalitis syndrome, severe influenza-like illness (ILI), or meningitis during March 3–May 24, 2014.

The case definition was met by 17 persons (11 acute encephalitis syndrome, 5 ILI, 1 meningitis). Clinical signs developed for the index case-patient on March 10 and for the last case-patient on April 21 (Figure 1). The case-fatality rate among those with acute encephalitis syndrome was 82%; no patient with ILI or meningitis died. Of acute encephalitis syndrome survivors, 1 experienced residual severe cognitive impairment, motor weakness, and ataxia, and the other experienced persistent ophthalmoplegia. Median incubation period for case-patients with known exposure was 8 days.

Table 1. Exposure and infection profile of henipavirus case-patients, Sultan Kudurat, Mindanao, Philippines, March 3–May 24, 2014

Clinical presentation	Slaughter and meat consumption	Meat consumption alone	Exposure to probably infected human	Uncertain exposure	Total
Acute encephalitis syndrome					
No. patients	3	3	4	1	11
Sex, M:F	3:0	3:0	4:0	1:0	11:0
Age , y	21, 32, 60	30, 51, 54	24, 29, 35, and 46	28	32 (median)
Incubation period	6, 8, 8	7, 10, 20	3–8. 6, 7, 8	Unknown	3–20
No. deaths	3	2	3	1	9
Influenza-like illness (n = 5) or meningitis (n = 1)					
No. patients	4	0	1	1	6
Sex, M:F	4:0	NA	1:0	0:1	5:1
Age, y	21, 23, 26, 39	NA	46	26	26 (median)
Incubation period, d	7, 9, 15, 15	NA	4	Unknown	4–15
No. deaths	0	NA	0	0	0

*NA, not applicable.

Of the 17 case-patients, a total of 7 (41%) had participated in horse slaughtering and horse meat consumption, and 3 (18%) had only consumed horse meat and had no history of slaughtering or meat preparation (Table 1). Five (29%) case-patients had been exposed to other human case-patients but not to any horses. Of these, 2 were health care workers from Isulan who did not visit the villages, had no contact with sick horses, and did not consume horse meat (Figure 1); they reportedly wore minimal personal protective equipment (gloves, face mask) during patient procedures, 2 cared for case-patients in their homes, and 1 helped transport a case-patient (who was producing substantial respiratory secretions) to a hospital.

During March 3–May 11, ten horse deaths were reported in the 2 villages (Figure 1); 2 were found dead, and all but 1 of the others showed neurologic signs (head tilting, circling, ataxia). Progression of clinical signs was rapid. Among other domestic animals, 4 cats that had eaten horse meat died within 5 days of their probable exposure date; 3 were found dead and the other exhibited terminal bleeding from the nose and/or mouth. A dog was found dead after eating horse meat, but the epidemiologic link is unknown.

Blood was collected from surviving suspected case-patients, contacts of human or horse case-patients, and several domestic animals (cats, buffalo, dogs, horses, pigs, goats). Retrospectively collected cerebrospinal fluid (n = 2) and serum (n = 7) samples from persons with suspected cases underwent further testing. No samples were available from affected horses.

Testing for a range of neurotropic pathogens was conducted at the Australian Animal Health Laboratory and the National Institute of Infectious Diseases (Japan). Test results were negative for all agents except henipaviruses.

To detect neutralizing antibodies against HeV and NiV, we used neutralization assays with infectious HeV and NiV (*14*) and pseudotyped vesicular stomatitis virus possessing NiV envelope proteins (*15*) (Table 2). Samples with positive results were subsequently tested by ELISA

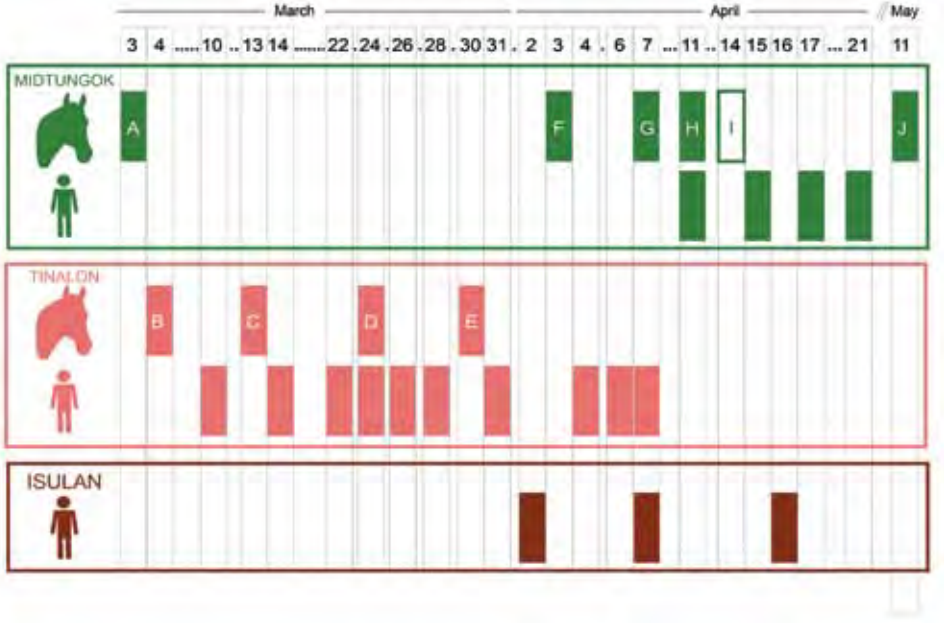

Figure 1. Temporal and geographic features of human and horse cases in 2 villages (Midtungok, Tinalon) and at the provincial referral hospital (Isulan), Philippines, 2014, by date of disease onset. Full rectangles represent cases based on case description. The empty rectangle (horse I) represents the horse death that did not fit the case description.

Table 2. Chronologic serologic test and nucleic acid detection results for 3 patients in NiV outbreak, Philippines, 2014*

Patient	Onset of clinical signs	Date of sample collection	IgM ELISA ratio	NiV SNT titer†	NiV SNT titer‡	Nucleic acid detection
1 (AES)	Apr 7	Apr 12	11.8	Neg	1:150	Pos (qPCR) from serum of Apr 12
		May 11	8.5	1:80	1:1,200	NA
		May 22	6.5	1:40	1:950	NA
2 (AES)	Apr 7	Apr 15	13.2 (6 AM), 12.9 (3 PM)	1:10	1:200	Pos (NGS) from CSF of Apr 12
		May 8	11.3	1:80	1:2,600	NA
		May 21	9.1	1:20	1:1,800	NA
3 (ILI)	Apr 2	May 21	5	1:40	1:420	NA

*The cutoff for the IgM NiV ELISA is a ratio of 2, for SNT using infectious NiV is ≥1:4, and for pseudotype-based SNT is 1:80. All samples were serum except for the sample tested by NGS, which was CSF. AES, acute encephalitis syndrome; CSF, cerebrospinal fluid; ILI, influenza-like illness; NA, not applicable; Neg, negative; NGS, next-generation sequencing; NiV, Nipah virus; pos, positive; qPCR, quantitative PCR for NiV; SNT, serum neutralization test.
†Test used infectious NiV.
‡Test used pseudotyped vesicular stomatitis virus.

for IgM against NiV. Neutralizing antibodies against NiV and correspondingly lower neutralizing antibody titers against HeV were found for 3 patients. IgM against NiV was also detectable in these same 3 patients. The pattern of neutralizing antibodies and IgM in acute-phase and convalescent-phase serum samples is evidence of recent exposure to a henipavirus. A serum sample from 1 of these patients (obtained 5 days after clinical sign onset) was also positive by real-time PCR for NiV, and a single-sequence read (71 bp) of the P gene of NiV was detected from a

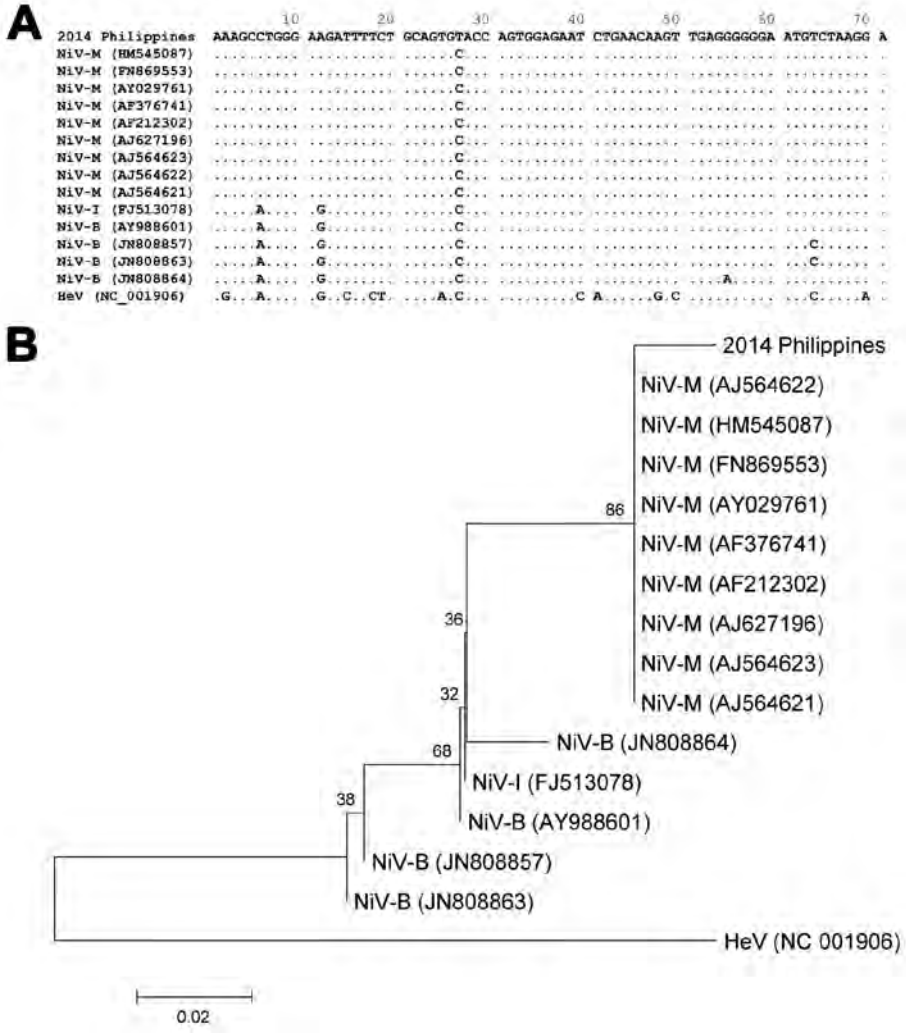

Figure 2. Alignment (A) and phylogenetic relationship (B) of partial phosphoprotein gene sequences (71 mer) of henipaviruses, including the fragment obtained by next-generation sequencing from a patient in Philippines (2014 Philippines). The alignment was conducted by using the MUSCLE program (http://www.ebi.ac.uk/Tools/msa/muscle/), and the phylogenetic tree from these data was constructed by using the neighbor-joining method. The optimal tree with sum of branch length equal to 0.23440320 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown. The phylogenetic tree is drawn to scale; branch lengths in the same units as those of the evolutionary distances are used to infer the phylogenetic tree. The scale bar represents 0.02 substitutions per site. The evolutionary distances were computed by using the Kimura 2-parameter method and are presented as number of base substitutions per site. The analysis involved 16-nt sequences. All positions containing gaps and missing data were eliminated. The final dataset contained 71 positions. Evolutionary analyses were conducted by using MEGA6 (http://www.megasoftware.net). The accession numbers of each sequence are shown for the viruses. HeV, Hendra virus. NiV-B, Nipah virus Bangladesh strain; NiV-I, Nipah virus Indian strain; NiV-M, Nipah virus Malaysian strain.

MiSeq (http://systems.illumina.com/systems/miseq.html) next-generation sequencing run of a cerebrospinal fluid sample from another of these patients (Figure 2). This short segment had 99% nt identity with NiV isolates from Malaysia and 94%–96% identity with NiV isolates from Bangladesh. Further attempts to amplify additional genome and isolate the virus were unsuccessful. The short-read archive has been deposited in the DNA Data Bank of Japan (accession no. DRA002637). All serum samples from 4 dogs were positive for neutralizing antibodies against NiV. NiV neutralizing antibodies were not detected in samples from animals of any other species.

Conclusions

Clinical presentations, epidemiologic findings, and serologic results suggest that the virus causing this outbreak was a henipavirus. It was most likely NiV or a virus that is antigenically and genetically closely related to NiV.

Epidemiologic data suggest that the most common route of virus transmission to humans was direct exposure to infected horses, contact with contaminated body fluids during slaughtering of sick horses, and/or consumption of undercooked meat from infected horses. However, for at least 5 cases, clinical and epidemiologic evidence suggest direct human-to-human virus transmission. No protective equipment was used by those who cared for case-patients in the home, and health care workers used gloves and a face mask but not eye protection. The evidence of human-to-human transmission in this outbreak confirms the need for preventative measures in home care and health care settings.

Although the source of the horse infections is unclear, on the basis of the known ecology of henipaviruses, the most likely source is fruit bats (family *Pteropodidae*) (*10*). Bats belonging to this family were reported near at least 1 of the 2 villages.

Ongoing surveillance in the area and neighboring regions is needed to help with prompt response to future outbreaks. Activities should include accurate and rapid diagnosis of new outbreaks, investigation of risk factors associated with spillover and virus transmission, implementation of control measures, and further characterization of the virus involved.

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Ascariasis in Humans and Pigs on Small-Scale Farms, Maine, USA, 2010–2013

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Ascaris is a genus of parasitic nematodes that can cause infections in humans and pigs. During 2010–2013, we identified 14 cases of ascariasis in persons who had contact with pigs in Maine, USA. *Ascaris* spp. are important zoonotic pathogens, and prevention measures are needed, including health education, farming practice improvements, and personal and food hygiene.

Ascaris spp. are parasitic nematodes whose eggs can remain infective in the environment for years. Ascariasis, infection with *Ascaris* spp., results from ingestion of infective eggs (*1*). *A. lumbricoides* nematodes are among the most prevalent human parasites worldwide, infecting >1 billion persons globally (*2*). Most human *Ascaris* infections are asymptomatic, but symptoms can include acute lung inflammation, abdominal distension and pain, and intestinal obstruction (*2*). Pigs are infected with *A. suum* nematodes, and symptoms can include coughing or thumping, liver damage, impaired growth, and increased susceptibility to other infections (*3*).

The overall extent of ascariasis in human and pigs in the United States, and in Maine, is unknown because the infection is not nationally notifiable or reportable at the state level. Ascariasis and other soil-transmitted helminth infections were highly prevalent in the southern United States and Appalachia as recently as the 1980s and were largely attributable to poor sanitation and poverty (*4*,*5*). Less is known about ascariasis prevalence elsewhere in the United States, including the Northeast, but infection has been presumed to be uncommon.

Experimental cross-transmission studies have demonstrated that *A. lumbricoides* can infect pigs and that *A. suum* can infect humans (*3*,*6*). Studies have also indicated that pigs are the main source of human *Ascaris* infections in areas considered to have no or low *Ascaris* prevalence (*7*–*9*). We describe 14 human cases of ascariasis associated with contact with pigs at 7 farms in Maine, USA, during 2010–2013 (*10*). In particular, we highlight an investigation at 1 farm (farm X).

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The Study

The 14 human ascariasis cases were reported by human and animal health care providers to the Maine Department of Health and Human Services (DHHS) or the Maine Department of Agriculture, Conservation and Forestry. Maine DHHS staff interviewed patients from each farm. Patients with confirmed infection had excreted in stool ≥1 worm that was subsequently laboratory-identified as *Ascaris* sp. Patients with probable infection reported excreting ≥1 worm in stool and were epidemiologically associated with a confirmed patient. Patients with suspected infection were persons with symptoms consistent with larval migration who had been on the same implicated farm as a patient with confirmed infection or persons who excreted ≥1 worm in stool without laboratory confirmation or association with a patient with confirmed illness.

The Figure displays geographic information regarding the 14 human cases (8 confirmed, 4 probable, and 2 suspected) from 7 unrelated small-scale farms in 6 counties in Maine. Three of the 7 farms raised organic vegetables. Workers or residents did not rotate among farms, but all 14 case-patients reported contact with pigs. Ten (71%) patients reported no international travel history. Of the 4 patients with a history of international travel, 2 reported receiving previous treatment for parasites. The Table shows the location of farms where infections were found, the laboratory results, and case classifications made during April 2010–March 2013. Patients were 1–53 years of age (median 25 years); 93% were female. The 3 pediatric patients were children who resided at 2 of the farms.

In October 2012, Maine DHHS conducted a site visit of farm X. A pooled fecal specimen was collected from 10 pigs. A worm specimen from a person was sent to the Centers for Disease Control and Prevention (CDC) for identification. Pooled pig feces and pig and human worm specimens were sent to private, university, and CDC laboratories for identification.

Figure. Locations of human ascariasis cases in Maine, USA, 2010–2013.

Farm X grew and sold organic vegetables and raised conventional and organic livestock, including dairy cows, laying hens, sheep, and pigs. Among 12 persons who worked at farm X during the fall of 2012, a total of 3 ascariasis cases occurred; all patients had gastrointestinal illness onsets after May 2012 and were treated with albendazole. Of these 3 patients, 2 reported travel in Africa during the previous year. One of these patients, whose *Ascaris* spp. infection was confirmed, had 3 stool samples reportedly test negative for parasites in February 2012 before returning to United States. The other patient who reported international travel and who had probable *Ascaris* spp. infection was reportedly treated for unspecified parasites in December 2011 while abroad. The third patient, who also had probable *Ascaris* spp. infection, reported travel in Asia within the previous 2 years but was not previously screened or treated for parasitic infections.

In October 2012, worms were recovered from 5 of 10 pigs from farm X at slaughter. Farm X had purchased these pigs as piglets from a local supplier in May 2012. During July 2012, the animals were treated for cough with dichlorvos. Farm X periodically rotates pig pens and vegetable gardens to different locations. These pigs were penned where pigs had been raised 1.5 years previously, in an area ≈15 feet from active vegetable plots. A mixture of hay used as bedding material for pigs, and pig manure was used as fertilizer for growing vegetables. Three handwashing stations were observed at farm X.

At farm X, we detected *Ascaris* eggs in pooled feces by zinc sulfate and sugar flotation testing methods. Human and pig worm specimens collected at farm X were confirmed as *Ascaris* spp. at CDC. We were unable to determine if pigs from any of the 7 affected farms had a source in common (e.g., common swine stock or breeder).

Conclusions

We determined that direct or indirect exposure to pigs was the single common factor in all 14 cases of human ascariasis we investigated. At farm X, where detailed information was available regarding pig husbandry, the timing of illness among the farm workers was consistent with acquisition of *Ascaris* infection from pigs. Cross-transmission of *Ascaris* infections between humans and pigs likely occurred on these farms.

Ascariasis occurred at multiple locations in Maine where farm workers had no history of travel to parts of the world where transmission occurs, strengthening our position that pigs introduced infection. We believe that *Ascaris* eggs persisted in farm soil for extended periods, which led to ongoing transmission (*11*). Laboratory rRNA analyses of the pig and human isolates contribute to a growing body of evidence that *A. lumbricoides* and *A. suum* are genetically very closely related and might, in fact, be a single species (*12–14*).

Certain farm practices might have contributed to human exposure to *Ascaris* eggs, including, as noted at farm X, use of pig manure as fertilizer, use of pig bedding for compost, and location of pig pens near where produce is grown. None of the 7 farms we investigated managed pigs according to organic farming standards, and pigs were not regularly dewormed. The human infections occurred on Maine farms with limited numbers (<30) of pigs, where farmers might be unaware of their risk for acquiring ascariasis. Maine does not have a wild pig population; therefore, wild pigs were not a potential source of infection or environmental contamination.

Recommendations to reduce transmission of *Ascaris* spp. nematodes include keeping pig pens separate from vegetable fields and avoiding use of pig manure for fertilizer, especially on produce. Ideally, farms should have dedicated equipment for handling animal waste and stall cleaning. Farm workers should wash hands before and after contact with pigs, pig waste, or soil contaminated with pig waste. Because *Ascaris* eggs can remain viable for extended periods in soil, raw produce should be washed

Table. Sample source information, results of laboratory testing, and case classification for *Ascaris* spp. nematode infections in humans and pigs on 7 farms, Maine, USA, 2010–2013*

Farm location and source	Sample type and year collected	Testing laboratory	Testing result	Case classification
Aroostook County				
Woman, age 25 y	NA			Suspected
Kennebec County				
Woman, age 21 y	Worms shed in feces, 2010	Private laboratory	*Ascaris* sp.	Confirmed
Woman, age 26 y	Worms shed in feces, 2010	Private laboratory	*Ascaris* sp.	Confirmed
Woman, age NA	NA			Suspected
Lincoln County†				
Boy, age 3 y	Worms shed in feces, 2012	CDC	*Ascaris* sp.	Confirmed
Woman, age 31 y	Worms shed in feces, 2012	CDC	*Ascaris* sp.	Confirmed
Woman, age 29 y	**Worms shed in feces, 2012**	**CDC**	***Ascaris* sp.**	**Confirmed**
Woman, age 36 y	**NA**			**Probable**
Woman, age 25 y	**NA**			**Probable**
Farm X pigs	**Pooled pig feces, 2012**	**Cornell**	***A. suum* eggs**	**NA**
Farm X pigs at slaughter	**5 worms collected from pigs, 2012**	**CDC**	***Ascaris* sp.**	**NA**
Piscataquis County				
Woman, age 53 y	Worms shed in feces, 2013	CDC	*Ascaris* sp.	Confirmed
Pig from farm	Pooled pig feces, 2013	Cornell	*A. suum* eggs	NA
Somerset County				
Woman, age 24 y	Worms shed in feces, 2012	Private laboratory	*Ascaris* sp.	Confirmed
Waldo County				
Woman, age 29 y	Worms shed in feces, 2012	CDC	*Ascaris* sp.	Confirmed
Girl, age 4 y	NA			Probable
Girl, age 2 y	NA			Probable

*Boldface indicates samples from farm X. CDC, Centers for Disease Control and Prevention; Cornell, Cornell University Animal Health Diagnostic Center; NA, not available.
†Two farms were located in Lincoln County.

thoroughly before consumption. For optimal animal health, pigs should be dewormed before introduction to the farm and should be regularly dewormed, and humans and pigs should be treated concurrently when human cases occur. Preventing *Ascaris* infections requires an integrated, One-Health approach that addresses on-farm practices, animal husbandry, and health education efforts.

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Dr. Miller was an Epidemic Intelligence Service officer with CDC, assigned to the Maine Center for Disease Control and Prevention in Augusta, at the time of this study. Her primary research interest is HIV, and she has additional experience working in both infectious and chronic disease epidemiology.

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Potentially Novel *Ehrlichia* Species in Horses, Nicaragua

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Ehrlichia sp. DNA was amplified from 4 *Ehrlichia*-seroreactive horses from Mérida, Nicaragua. Sequencing of 16S rDNA, *sodB,* and *groEL* genes indicated that the bacterium is most likely a novel *Ehrlichia* species. The tick vector and the potential for canine and human infection remain unknown.

Worldwide, ehrlichioses are considered emerging infectious diseases of animals and humans. Transmitted by ticks, ehrlichae are obligate intracellular, gram-negative bacteria that infect animals and humans (*1*). Recognized species include *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, and *E. ruminantium* (*1*). Other species identified in North America are Panola Mountain (*2*) and the *E. muris*–like agent (*3*). Two recently identified new species are *Ehrlichia* sp. AvBat, isolated from *Argas vespertilionis* ticks in France (*4*), and *E. mineirensis*, isolated from hemolymph of *Rhipicephalus microplus* ticks in Brazil (*5*).

Although studies from Brazil and Oklahoma (USA) have documented reactivity to *Ehrlichia* spp. (*6*,*7*) in horse serum, no reports have documented isolation or PCR detection of *Ehrlichia* spp. infection in horses worldwide. In North America, cervids are reservoir hosts for *E. chaffeensis*, which after tick transmission causes monocytic ehrlichiosis in humans (*1*). Although equids are not known hosts for *E. chaffeensis*, bacterial DNA has been amplified from ticks (*Dermacentor nitens* and *Amblyomma cajennense*) collected from horses in Panama (*8*). *Anaplasma phagocytophilum* and *Borrelia burgdorferi*, causes of granulocytic anaplasmosis and borreliosis (Lyme disease), respectively, are transmitted by *Ixodes scapularis* and *I. pacificus* ticks in North America and infect cats, dogs, horses, and humans.

In Mérida, Nicaragua, the potential for infection of horses by tickborne pathogens is a concern because of the horses' often poor body condition and heavy tick infestations. In 2013, to determine exposure of equids to >1 tickborne organism, visiting veterinary students collected blood samples from horses.

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The Study

With approval from the Oregon State University Animal Care and Use Committee (Animal Care and Use Proposal no. 4329), blood samples were collected from 92 horses being evaluated for medical conditions (e.g., anorexia, weight loss, lameness, administration of endoparasiticides and ectoparasiticides) or before elective surgery (e.g., castration, wound repair) at the clinic in Mérida from August 28 through September 4, 2013. After jugular venipuncture, 6 mL of blood was collected into EDTA tubes.

Each whole blood sample was tested for antibodies against *Anaplasma* spp. (*A. phagocytophilum* and *A. platys*), *B. burgdorferi* sensu stricto, and *Ehrlichia* spp. (*E.canis*, *E. chaffeensis*, and *E. ewingii*) by using the ELISA-based assay SNAP 4DxPlus (IDEXX Laboratories, Inc., Westbrook, ME, USA) according to the manufacturer's instructions (*9*). The assay does not use a host species–specific conjugate and can therefore be used in research to screen mammals other than dogs. According to assay results, 51 (55%) horse serum samples were *Ehrlichia* spp. seroreactive. One sample was *B. burgdorferi* seroreactive, whereas none were *Anaplasma* spp. seroreactive.

The 51 *Ehrlichia* spp.–reactive serum samples were subsequently stored at 28°C for up to 1 week during transport to the United States. For PCR testing, samples were shipped (US Department of Agriculture import permit no. 13846) to the Intracellular Pathogens Research Laboratory, Center for Comparative Medicine and Translational Research, North Carolina State University, College of Veterinary Medicine. DNA extraction from 200 mL of EDTA-anticoagulated whole blood was performed by using a QIAsymphony DNA Mini Kit (QIAGEN, Valencia, CA, USA; catalog no. 931236). Previously described PCRs were used to amplify a 420-bp fragment of the *16S* rRNA gene, a 620-bp fragment of the *GroEL* gene, and a 300-bp fragment of the *Ehrlichia* spp. *sodB* gene (*2*,*10*,*11*). A larger, 600-bp, fragment of the *sodB* gene was amplified from 1 sample that was positive by PCR by using the following unpublished primers: sodbEhrl600-F 5′-ATGTTTACTTTACCTGAACTTCCATATC-32 and sodbEhrl600-R 5′-ATCTTTGAGCTGCAAAATCCCAATT-32. Positive (*Anaplasma* or *Ehrlichia* spp. plasmid DNA) and negative (RNase-free molecular-grade water and a DNA extraction control consisting of uninfected canine genomic DNA) controls were used for each assay. Amplified DNA was sequenced directly

by GENEWIZ, Inc. (Research Triangle Park, NC, USA), and alignments were compared with those of GenBank sequences by using AlignX software (Vector NTI Advance version 11.5; Invitrogen, Carlsbad, CA, USA).

Of the 51 samples tested by *16S* rDNA PCR, the rDNA amplicon sequences were identical for 4 (8%). Sequence comparisons of the amplified products with *Ehrlichia* spp., *Anaplasma* spp., and *Neorickettsia risticii* sequences in GenBank are summarized in the Table. Identical *GroEL* and *sodB* DNA sequences were amplified from 3 of 4 horses.

Conclusions

According to serologic, PCR amplification, and DNA sequencing results, tick-infested horses in Mérida, Nicaragua, might be infected with a potentially novel *Ehrlichia* species. Initial serologic screening with the rapid ELISA indicated that exposure to ≥1 *Ehrlichia* species is common among horses in Nicaragua (55%). For dogs, SNAP 4Dx-Plus results can be positive after exposure to *E. canis*, *E. chaffeensis*, *E. ewingii*, and potentially *E. muris* and Panola Mountain ehrlichiae *(2*,*3*,*6)*. Before this study, equine exposure to *A. phagocytophilum* (previous designation *E. equi*) in Nicaragua was considered more likely because equine exposure has occurred in Guatemala; this rickettsial organism is pathogenic for cats, dogs, horses, and humans (*12*). However, in North America, *A. phagocytophilum* is transmitted by *I. scapularis* and *I. pacificus* ticks, which have not been reported in Nicaragua. In future studies of horses in Nicaragua, ticks will be collected for identification. In Guatemala, *R. microplus* and *A. cajennense* ticks were the predominant species found on cattle, whereas *D. nitens* and *A. cajennense* ticks were most commonly found on horses (*12*). Of note, in Guatemala, tick infestation levels were substantially higher and body condition scores lower for horses than for cattle. Also, cattle were exposed to an agent with serologic cross-reactivity and close genetic relatedness to *E. ruminantium.*

The partial *16S* rDNA sequences obtained from these horses most likely represent a novel species of *Ehrlichia*. This conclusion is further supported by sequence analysis of 2 protein-coding genes, *sodB* and *groEL*. Partial sequences from *sodB* and *groEL* genes demonstrated similarity to *Ehrlichia* spp. sequences found in GenBank, but they were not 100% identical to any sequences deposited to date. When *16S* rDNA for rickettsiae are compared, Fournier et al. recommend that gene homology for organisms of identical species and genus be 99.8% and 98.1%, respectively (*13*). Although identical species are typically defined as being >99% identical with a reference sequence, the percentage identity needed to define a separate species is debated, ranging from 97% to 99.5% (*14*). Calculation of values can be based on alignment methods, reference databases, and number of basepairs in the sequence. Fournier et al. recommend that for novel species identification and rickettsiae classification, protein-coding genes should be used, specifically the 4 protein-coding genes *gltA*, *ompA*, *ompB*, and gene D in addition to the *16S* rRNA gene. To date, *16S* rDNA sequences or whole-genome sequencing have been used to classify *Ehrlichia* species and strains. Collectively, and as depicted in the Figure (in which phylogenetic alignment trees for all 3 genes tested in this study were constructed by using reference sequences from representative members of the genera *Anaplasma*, *Ehrlichia*, and *Neorickettsia*), the genetic findings in this study support infection of horses in Nicaragua with a novel *Ehrlichia* species. To confirm this possibility, future efforts will focus on cell culture isolation of the *Ehrlichia* organism from horses in Nicaragua.

Vectorborne pathogens can infect any host species bitten by infected ticks. At least 4 *Ehrlichia* species have been implicated as being pathogenic for canids and humans (*1*–*3*). Thus, future studies should also determine whether dogs, other animals, and humans in Nicaragua are exposed to and infected with this potentially novel *Ehrlichia* species.

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Table. Base pair similarities for DNA sequences of novel *Ehrlichia* species obtained from horses in Nicaragua in 2013*

Bacteria	GenBank accession nos.†	Gene, no. positive/no. tested (%)		
		16S rRNA	*GroEl*	*SodB*
E. ruminantium	CR925678	361/374 (96.5)	534/590 (90.5)	508/599 (84.8)
E. canis	CP000107	356/374 (95.2)	540/590 (91.5)	496/599 (82.8)
E. chaffeensis	CP000236	360/374 (96.2)	529/590 (89.7)	491/599 (82.0)
E. ewingii	NR_044747, AF195273, KC778986	352/374 (94.1)	530/590 (89.8)	241/306 (78.8)
A. marginale	CP006847	339/374 (90.6)	417/590 (70.8)	348/599 (58.1)
A. phagocytophilum	CP006618	348/374 (93.0)	439/590 (74.4)	367/599 (61.2)
N. risticii	CP001431	306/374 (81.8)	395/590 (66.9)	358/599 (59.8)

*Sequences for partial *16S* rRNA, *GroEl*, and *SodB* genes are compared with GenBank database sequences from *Ehrlichia ruminantium, E. canis, E. chaffeensis*, *E. ewingii, Anaplasma marginale, A. phagocytophilum*, and *Neorickettsia risticii.*
†GenBank sequence accession numbers for *16S* rRNA, *GroEl,* and *SodB* for *Ehrlichia* sp. from horses in Nicaragua are KJ434178, KJ434179, and KJ434180, respectively.

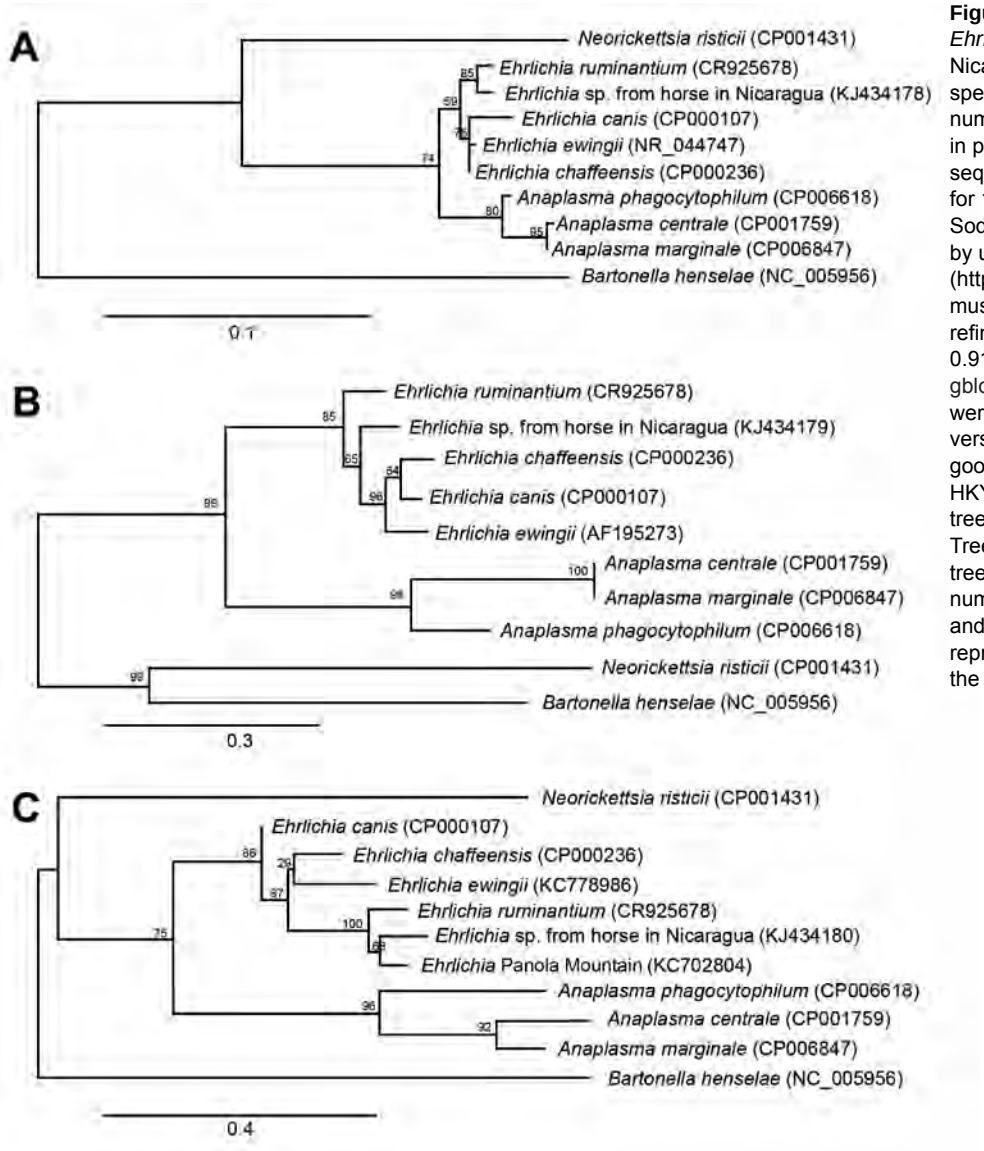

Figure. Phylogenetic trees of *Ehrlichia* sp. from horses in Nicaragua and selected bacterial species (GenBank accession numbers for reference sequences in parenthesis) based on partial sequences from genes coding for 16SrRNA (A), GroEL (B), and SodB (C). Sequences were aligned by using MUSCLE version 3.7 (http://www.ebi.ac.uk/Tools/msa/muscle/), and alignments were refined by using Gblocks version 0.91b (http://www.idtdna.com/gblocks.com). Phylogenetic trees were constructed by using PhyML version 3.0 aLRT (http://code.google.com/p/phyml/) under the HKY85 model, and the resulting trees were rendered by using TreeDyn version 198.3 (http://www.treedyn.org/). Scale bars indicate number of substitutions per site, and the numbers in the branches represent percentage support of the node.

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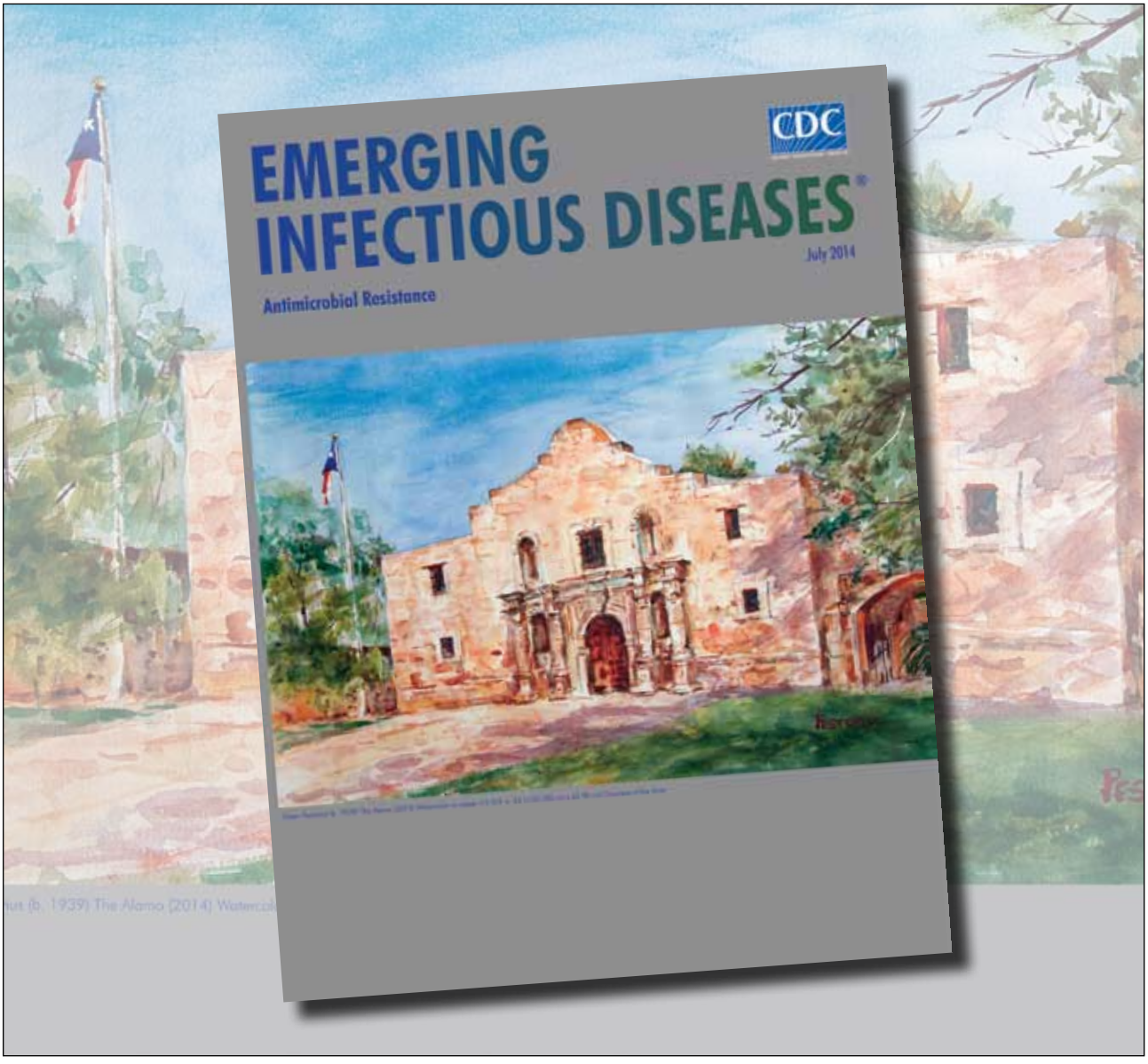

Neisseria meningitidis ST-11 Clonal Complex, Chile, 2012

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Serogroup W *Neisseria meningitidis* was the main cause of invasive meningococcal disease in Chile during 2012. The case-fatality rate for this disease was higher than in previous years. Genotyping of meningococci isolated from case-patients identified the hypervirulent lineage W:P1.5,2:ST-11, which contained allele 22 of the *fHbp* gene.

Neisseria meningitidis (meningococcus) is the causative pathogen of invasive meningococcal disease (IMD), which includes a set of infectious syndromes, mainly meningitis or meningococcemia (septicemia) and, less commonly, pneumonia or other infections (*1*). Humans are the only reservoir for meningococcus, which usually colonizes the upper respiratory tract of ≈8%–25% of persons (*1*).

In Chile, the incidence of IMD decreased steadily during 2000–2012 from 3.6 to 0.7 cases/100,000 inhabitants (*2*). However, deaths from this disease have not followed this trend; the case-fatality rate increased from 8.9% in 2009 to 14.1% in 2010, 14.7% in 2011, and 27.0% in 2012 (*2*,*3*). During this period, the distribution of meningoccal serogroups has changed. There has been a large increase in frequency of serogroup W meningococci, which has replaced serogroup B as the most common serogroup.

A total of 101 cases, 57 culture-confirmed: 42/57 (73.7%) serogroup B and 1/57 (1.8%) serogroup W were reported in 2009; 78 cases: 56 culture-confirmed 36/56 (64.3%) serogroup B and 6/56 (10.7%) serogroup W were reported in 2010; 73 cases: 63 culture-confirmed, 32/63 (50.8%) serogroup B and 22/63 (34.9%) serogroup W were reported in 2011; and 133 cases: 103 culture-confirmed, 38/103 (36.9%) serogroup B and 60/103 (58.3%) serogroup W were reported in 2012 (*2*–*4*). We conducted this study to determine whether W meningococci belonged to a hypervirulent genetic lineage of the ST-11 clonal complex (CC).

The Study

A national epidemiologic program for surveillance and control of IMD is conducted by the Department of Epidemiology of the Ministry of Health of Chile. Every national health care center must report suspected cases of IMD and send bacterial isolates to the Institute of Public Health of Chile (Santiago, Chile) or send cerebrospinal fluid samples when cultures have shown negative results. IMD cases are defined by clinical signs and symptoms (neck stiffness, altered state of consciousness, rash, meningeal irritation) and confirmed by isolation of *N. meningitidis* from cerebrospinal fluid, blood, or another sterile body fluid or tissue. Each case is coded according to the International Classification of diseases, 10th Revision, as meningitis (code A39.0), meningococcemia (A39.2), Waterhouse-Friderichsen syndrome (A39.1), other meningococcal infections (A39.8), and unspecified meningococcal infections (A39.9).

In 2012, a total of 32 health care centers located throughout Chile reported 133 IMD cases. Of these cases, 103 were laboratory confirmed by bacterial isolation and biochemical identification (*3*). Serogroup was determined by slide agglutination with polyclonal antibodies. Genosubtyping was conducted by amplifying and sequencing variable regions 1 and 2 of the *porA* gene as described by Russell et al. (*5*) Variants were defined by reviewing the *Neisseria PorA* typing database (http://pubmlst.org/neisseria/PorA/). Sequence types (STs) were determined as described by Maiden et al. (*6*) on the basis of housekeeping genes *abcZ*, *adk*, *aroE*, *fumC*, *gdh*, *pdh*, and *pgm*. Sequences were compared with those in the *Neisseria* locus/sequence definition database (http://pubmlst.org/), and STs and CCs were assigned. The nomenclature used in this report, when appropriate, is serogroup: genosubtype: CC.

The *fHbp* genetic variant was identified as described by Brehony et al. (*7*). Allele numbers were assigned by querying a public database (*Neisseria* Factor H binding protein sequence typing; http://pubmlst.org/neisseria/fHbp/). Genotyping with higher resolution was conducted by using pulsed-field gel electrophoresis (PFGE) and restriction endonuclease *Spe*I. Electrophoretic profiles were analyzed by using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). A PFGE pattern was considered unique when ≥1 DNA bands in the electrophoretic migration profile differed from each other. A code was assigned to each pattern. To establish the magnitude of associations, we cross-tabulated data and calculated odds ratios (ORs) by using Med Calc software version

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12.4.0.0 (http://www.medcalc.org/). The 95% CI was established, and p values <0.05 were considered significant.

Serogroup B meningococci were isolated from 38 (36.9%) case-patients, and serogroup W meningococci were isolated from 60 (58.2%) case-patients (*4*). Serogroups C and Y meningococci were rarely isolated (3 and 2 isolates, respectively). Multilocus sequencing typing showed serogroup B isolates belonged mainly to ST-32 and ST-41/44 CCs, including 4 and 6 STs respectively. Of 60 W isolates, 98% belonged to ST-11 CC (Table 1). This finding is consistent with the serogroup of CCs found in W meningococci isolated in Chile during 2010 (4/4 W isolates analyzed) and 2011 (19/21 W isolates) (*8*). Among this CC, 3 STs were identified, of which ST-11 was the most common (Table 1).

Genosubtyping of W:ST-11 strains obtained during 2012 indicated that 58/60 strains belonged to genosubtype P1.5,2 (Table 2). In addition, sequence analysis of the *fHbp* gene identified allele 22 as the most common variant; it was present in 58 (96.7%) of 60 strains that belonged to the W serogroup (Table 2). Allele 22 of *fHbp* was not detected in strains belonging to other serogroups, which indicated a strong association with W meningococci. These results indicated that most IMD cases reported during 2012 in Chile were caused by a hypervirulent genetic lineage of *N. meningitidis* serogroup W.

Serogroup W meningococci W:P1.5,2:ST-11 with allele 22 of *fHbp* were isolated from samples of 12 of 42 meningitis case-patients reported during 2012 and from 38 of 51 samples from meningococcemia case-patients. This association with meningococcemia cases was significant (OR 5.5, 95% CI 2.4–12.9, p = 0.0001). Overall, of 103 IMD case-patients in this study, 22 died. W:P1.5,2:ST-11 meningococci carrying allele 22 of *fHbp* were isolated from most patients with lethal cases. However, this association was not significant (16 of 22 deaths; OR 2.5, 95% CI 0.9–6.9, p = 0.08).

Analysis of W:P1.5,2:ST-11 meningococci by PFGE identified 9 electrophoretic patterns (Table 2). PFGE showed that most hypervirulent W:ST-11 clones were closely related to each other. Meningococci with a Cl-Nm-Spe-031 pattern caused most meningococcemia cases (Table 2). However, this association was not significant (OR 1.8, 95% CI 0.59–5.38, p = 0.31). Of 16 lethal cas-

Table 1. Clonal complexes and sequence types identified in main serogroups of *Neisseria meningitidis* isolated in Chile, 2012*

Serogroup, clonal complex	No. (%) isolates	Sequence type
B, n = 38		
ST-32	17 (44.7)	NA
	13	32
	2	3822
	1	7780
	1	9918
ST-41–44	20 (52.6)	NA
	14	44
	1	315
	1	8528
	2	9233
	1	9354
	1	10127
ST-461	1 (2.6)	NA
	1	461
W, n = 60		
ST-11	59 (98.3)	NA
	51	11
	7	1025
	1	2961
ST-22	1 (1.7)	NA
	1	184

*NA, not applicable.

Table 2. Characteristics of *Neisseria meningitidis* serogroup W isolates from 60 patients with invasive meningococcal disease, Chile, 2012*

Characteristic	Genotype				
	W:P1.5,2:ST-11, n = 58			W:P1.5,2–53:ST-11, n = 1	W:P1.18–1.3:ST-22, n = 1
Clinical outcome†	A39.0, n = 12	A39.2, n = 39	Other, n = 7	A39.2, n = 1	A39.0, n = 1
fHbp gene allele					
16, n = 1	0	0	0	0	1
19, n = 1	0	1	0	0	0
22, n = 58	12	38	7	1	0
PFGE pattern					
Cl-Nm-Spe-030, n = 7	1	5	1	0	0
Cl-Nm-Spe-031, n = 33	5	24	4	0	0
Cl-Nm-Spe-044, n = 2	0	2	0	0	0
Cl-Nm-Spe-046, n = 8	2	5	1	0	0
Cl-Nm-Spe-083, n = 1	0	0	0	0	1
Cl-Nm-Spe-084, n = 3	2	1	0	0	0
Cl-Nm-Spe-085, n = 2	1	1	0	0	0
Cl-Nm-Spe-086, n = 1	1	0	0	0	0
Cl-Nm-Spe-087, n = 1	0	0	1	0	0
Cl-Nm-Spe-088, n = 1	0	1	0	0	0
Cl-Nm-Spe-100, n = 1	0	0	0	1	0

*PFGE, pulsed-field gel electrophoresis.
†Codes from International Classification of Diseases, 10th Revision. Clinical outcomes: meningitis (A39.0); meningococcemia (A39.2); Other: Waterhouse-Friderichsen syndrome (A39.1); meningococcemia unspecified (A39.4); other meningococcal infections (A39.8) and nonspecified invasive meningococcal disease (A39.9).

es reported that were associated with W meningococci, 9 were caused by strains with the Cl-Nm-Spe-031 pattern, 3 by strains with the Cl-Nm-Spe-030 pattern, 3 by strains with the Cl-Nm-Spe-046 pattern, and 1 by a strain with the Cl-Nm-Spe-085 pattern.

Conclusions

We showed that most W meningococci belonged to a hypervirulent genetic lineage of the ST-11 CC. Hypervirulent serogroup W meningococci W:P1.5,2:ST-11, which has the *fHbp* gene allele 22, was the main cause of IMD in Chile during 2012. Its presence was associated with meningococcemia cases and partially accounted for more deaths during 2012 than in previous years. These isolates have a genetic profile similar to that of isolates from the first outbreak of IMD attributed to serogroup W, which affected the Hajj pilgrimage in Saudi Arabia in 2000, and to that of isolates from a larger outbreak in Burkina Faso in 2002 (*9*). However, these isolates from Chile have allele ID 22 of *fHbp* instead of alleles ID 9 or ID 23. This allele has also been found in a hypervirulent W:P1.5,2:ST-11 strain in Mali in 1994 (*10*), but we have not found more instances of its presence.

Some genotypes of these isolates have been detected in serogroup W strains obtained in previous years, specifically genosubtype (*8*), allele 22 of the *fHbp* gene (17 strains obtained in 2011), and specific PFGE patterns (9 strains were Cl-Nm-Spe-030, 4 were Cl-Nm-Spe-031, and 3 were Cl-Nm-Spe-046). These results indicate that hyperinvasive clones are circulating in Chile.

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Molecular Diagnosis of Cause of Anisakiasis in Humans, South Korea

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Anisakiasis in humans in South Korea has been considered to be caused exclusively by the larvae of *Anisakis simplex* sensu stricto and *Pseudoterranova decipiens.* Recently, however, DNA sequencing of larvae from 15 of 16 anisakiasis patients confirmed the cause to be *Anisakis pegreffii* infection. Molecular analysis should be performed for all extracted larvae.

Anisakiasis is a zoonotic nematode infection that causes acute and chronic gastrointestinal granulomatous disease in humans. For most patients, the causative agents are larvae of nematodes of the genera *Anisakis* and *Pseudoterranova*, and the source of infection is marine fish or squids harboring these larvae (*1*). Within 8–12 hours after infected fish are ingested, the larvae penetrate into the person's stomach or intestinal wall, causing acute abdominal pain, indigestion, nausea, and vomiting; pathologic findings are edema, hyperemia, and bleeding in the surrounding mucosa (*1*,*2*). The diagnosis is usually based on morphologic identification of the larvae or on histopathologic identification of sectioned larvae (*1*). However, molecular techniques have recently been developed as effective tools not only for the diagnosis of individual cases but also for studies of taxonomy and evolution of anisakid nematodes (*3*,*4*).

Anisakiasis in humans was first reported in the Netherlands; since then, it has been reported extensively in Japan (≈2,000 cases annually), South Korea (≈200 cases annually), and some European countries (≈500 cases annually) where people eat raw or undercooked fish (*1*,*2*). In the United States, up to 50 human cases are reported each year (*1*). Most infections in humans have been caused by *Anisakis simplex* sensu stricto and *Pseudoterranova decipiens* nematodes (*1*); however, since 1999, a few human infections with *Anisakis pegreffii* larvae (a sibling species of *A. simplex* s.s.), originally recovered from a Mediterranean monk seal (*5*), have been reported in Italy (*6*–*9*) and Japan (*10*,*11*). The larvae of *A. pegreffii* are morphologically distinguished, with difficulty, from those of *A. simplex* s.s. (both are *Anisakis* type I); however, molecular techniques can easily distinguish the 2 types of larvae (*3*,*4*).

In South Korea, *Anisakis* type I larvae recovered from humans and fish have been assigned to *A. simplex* s.s., on the basis of morphologic appearance (*1*,*12*). We performed molecular analyses of 26 *Anisakis* type I larvae recovered from 16 humans in South Korea by using DNA sequencing of the nuclear internal transcribed spacer (ITS) genes.

The Study

A total of 30 *Anisakis* type I larvae were removed from the stomach of 16 patients referred to the Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, Seoul, South Korea, from 2000 through 2013 (Table). Among them, 26 larvae were analyzed by DNA sequencing. All patients experienced acute gastric or abdominal discomfort, including epigastric pain and indigestion, and underwent gastroduodenoscopy. During the examinations, whitish nematode larvae were observed and extracted with biopsy forceps. Some larvae were preserved in 70% ethanol, and others were fixed in 10% formalin before being mounted on slides with glycerin jelly.

Total genomic DNA was extracted by using a DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany); nested PCR and nucleotide sequencing were performed on the ITS region (ITS1, 5.8S rRNA subunit, and ITS2) according to procedures reported previously (*13*). The PCR product was amplified by using the Cosmo Labopass X2 PCR Premix kit (Cosmo Genetech, Seoul, South Korea), and automated DNA sequencing was performed by Solgent Co., Ltd. (Daejeon, South Korea). Nucleotide sequences obtained were aligned by using the Geneious program, version 6.0.3 (Geneious Co., Wellington, New Zealand).

Of the 26 *Anisakis* larvae from 15 human patients, 25 showed 100% identity in the sequences of ITS region (244 bp, high-confidence variable positions) with those of the *A. pegreffii* sequence available in GenBank (accession no. AB277823), whereas their identity with *A. simplex* s.s. (accession no. AB277822) was 99.2% (Table). The remaining sample showed 100% identity with the sequences of *A. simplex* s.s. (accession no. AB277822) and 99.2% identity with those of *A. pegreffii* (accession no. AB277823). On the basis of these results, *A. pegreffii* nematode infection was diagnosed for 15 of the 16 patients, and *A. simplex* s.s. infection was diagnosed for only 1 patient.

Conclusions

Our results confirm the presence of *A. pegreffii* nematode infection in humans in South Korea, making this the third

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Table. Anisakiasis characteristics among 16 human patients, South Korea, 2000–2013*

Patient no.	Patient sex	Year of larvae recovery	No. larvae recovered	Clinical signs and symptoms	*Anisakis* larvae sequencing results (% identical sites)	
					A. simplex s.s.	*A. pegreffii*
1	M	2000	1	Abdominal pain, nausea	99.2	100
2	M	2000	1	Indigestion, vague gastric pain	99.2	100
3	M	2002	1	Abdominal pain, nausea, vomiting	99.2	100
4	F	2002	1	Abdominal pain and tenderness, anorexia	99.2	100
5	F	2003	1	Epigastric pain, nausea, vomiting	99.2	100
6	M	2003	1	Abdominal pain, indigestion	99.2	100
7	F	2003	1	Abdominal pain, nausea, diarrhea	99.2	100
8	F	2003	1	Abdominal pain, nausea, anorexia	99.2	100
9	F	2004	1	Epigastric pain, abdominal fullness	99.2	100
10	M	2005	1	Abdominal pain, nausea, vomiting	99.2	100
11	F	2005	1	Abdominal pain, indigestion, nausea, vomiting	99.2	100
12	M	2005	1	Abdominal pain, nausea, vomiting	99.2	100
13	M	2005	1	Epigastric pain, indigestion, nausea	99.2	100
14	M	2006	1	Abdominal discomfort, nausea	99.2	100
15	M	2012	1	Abdominal pain and tenderness	100	99.2
16	M	2013	15†	Abdominal pain and tenderness, indigestion, nausea, vomiting, anorexia	99.2	100

*All patients underwent gastroduodenoscopy, during which *Anisakis* larvae were removed with biopsy forceps.
†Of these 15 larvae, 11 were analyzed by use of molecular techniques.

country (after Italy and Japan) in which this infection in humans has been identified. This high proportion of *A. pegreffii* nematode infections in humans is surprising and suggests that most cases of anisakiasis in humans in South Korea may be caused by *A. pegreffii* rather than *A. simplex* s.s. larvae. To confirm the source of infection, molecular analyses of *Anisakis* larvae extracted from human patients are required in South Korea.

Human infection with *A. pegreffii* nematodes was first documented in Italy by use of PCR-based restriction fragment length polymorphism (PCR-RFLP) analysis (*6*). The second case was reported from Japan (*10*), in which 1 of 100 anisakid larvae extracted from patients in Kyushu and Hokkaido was identified by PCR-RFLP analysis as *A. pegreffii*. Then, in 2009, *A. pegreffii* nematode infection was diagnosed for 2 women in Italy by PCR-RFLP and sequencing of the 28S gene (*7*). In 2011, *A. pegreffii* DNA was extracted from a paraffin-embedded granuloma from a man in Italy (*8*). Also in Italy, 8 more *A. pegreffii* nematode infections in humans were reported in 2013 (*9*). Thus, to date, including the 15 cases reported here, a total of 28 cases of *A. pegreffii* nematode infections in humans have been documented in the literature.

The markedly high proportion of *A. pegreffii* nematode infections identified among patients in South Korea (25/26 larvae from 15/16 patients) was not expected because in Japan (Kyushu and Hokkaido), which are geographically close to South Korea, *Anisakis* larvae from humans are mostly *A. simplex* s.s. (99/100 larvae from 84/85 patients); only 1 larva was identified as *A. pegreffii* (*10*). This remarkable discrepancy between South Korea and Japan remains to be further investigated. However, it is of note that the species of *Anisakis* larvae detected in fish varied according to the 2 large localities of Japan; from northern Japan to the Pacific sides and from the Sea of Japan to the eastern China Sea sides (*14*). The former locality, such as Hokkaido and eastern Japan, showed more *A. simplex* s.s. than *A. pegreffii* larvae, whereas the latter locality (southwestern Japan), including Kyushu and Fukuoka (close to South Korea), showed more *A. pegreffii* than *A. simplex* s.s larvae (*14*). This finding might partly explain the discrepancy between the *Anisakis* larvae species that infect humans in South Korea and Japan.

When the pathogenic potential of *A. simplex* s.s. larvae for human patients was compared with that of *A. pegreffii* (*11*), it was found that *A. simplex* s.s. larvae had greater potential than *A. pegreffii* larvae to survive acidic gastric juice and to penetrate the human stomach, small intestine, and colon. However, further studies are needed to elucidate this finding.

Another clinicopathologic concern associated with anisakiasis in humans is the potential for *A. simplex* s.s.

and *A. pegreffii* larvae to elicit gastroallergic reactions. These reactions are characterized by urticaria on the arms and abdomen and by angioedema or anaphylaxis when the live parasite attempts to penetrate the gastric mucosa (*9*). We did not notice such allergic reactions in the patients reported here. However, because of an increasing tendency toward *Anisakis* nematode allergy among patients in South Korea (*15*), attention should be paid to this clinical feature.

Our study demonstrates the predominance of *A. pegreffii* over *A. simplex* s.s. nematode infection among humans with anisakiasis in South Korea. The study highlights the need to perform molecular analysis for each larva extracted from human patients in this country.

Ms. Lim and Mr. Jung are research associates in the Department of Parasitology and Tropical Medicine, Seoul National University College of Medicine, Seoul, South Korea. Their major research interest is molecular studies of foodborne zoonotic parasites, including anisakids, heterophyids, gymnophallids, and *Toxoplasma*.

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Streptococcus suis Infection in Hospitalized Patients, Nakhon Phanom Province, Thailand

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In Nakhon Phanom, Thailand, we identified 38 hospitalized patients with *Streptococcus suis* infection during 2006–2012. Deafness developed in 12 patients; none died. Thirty-five reported recent exposure to pigs/pork. Annual incidence was 0.1–2.2 cases/100,000 population (0.2–3.2 in persons ≥20 years of age). Clinicians should consider *S. suis* infection in areas where pig exposure is common.

Streptococcus suis, a zoonotic pathogen found primarily in pigs, can cause serious infection in humans. Most cases in human occur in Southeast Asia, where pig rearing is common (*1*). In a recent global review, Thailand had the second highest number of reported cases, accounting for 11% of all reported cases worldwide (*2*). In Thailand, the first 2 cases of *S. suis* in humans were reported in 1987 (*3*). From 1997 (when *S. suis* infection was first reportable) through 2010, a total of 692 cases were reported (0–207 per year); nearly half were from northern Thailand (*4*). The national annual crude incidence rate was 0–0.381 per 100,000 persons (Table 1). The objective of this study was to describe persons hospitalized with, and incidence of, *S. suis* infection in Nakhon Phanom during 2006–2012.

The Study

In 2003, the Thailand Ministry of Public Health and the US Centers for Disease Control and Prevention established hospital-based surveillance for community-acquired acute lower respiratory infections (ALRI) at all 12 acute-care hospitals in Nakhon Phanom Province (northeastern Thailand; population 761,623) (*5*). In 2005, surveillance was expanded to include bloodstream infections, supported by the addition of an automated blood culture system and improved microbiology capacity (*6*). Blood was collected for culture at clinician discretion but encouraged for all patients with ALRI and children <5 years of age who had sepsis. Incidence of pneumococcal bacteremia (all ages) and other bloodstream infections (children <5 years) was previously published (*6,7*). This work was considered public health surveillance and thus exempt from institutional review board review.

Blood put into a blood culture bottle was transported at 15–30°C within 24 hours to the provincial hospital laboratory and processed by using the BacT/ALERT 3D automated blood culture system (bioMérieux, Durham, NC, USA). To obtain at least 10 mL per adult patient, we divided specimens into 2 bottles (standard aerobic growth and enhanced growth of fastidious pathogens). Bottles that indicated positive growth were subcultured and processed by standard methods (*8*). All possible pathogens were confirmed at the National Institute of Health, Ministry of Public Health, by conventional biochemical tests (*9*). We serotyped *S. suis* isolates using PCR (*10*) and confirmed serotypes by coagglutination using rabbit antiserum (Statens Serum Institut, Copenhagen, Denmark). A case of *S. suis* was defined as illness in a person hospitalized in Nakhon Phanom who had blood culture–confirmed *S. suis* infection. Two physicians (K.P. and S.S.) reviewed the medical data retrospectively. Patients were interviewed by using a standard protocol (http://www.boe.moph.go.th/files/report/20100902_39823811.pdf). We calculated annual incidence using the estimated population as the denominator (http://www.nesdb.go.th/temp_social/pop.zip).

During 2006–2012, there were 56,983 blood cultures from 56,057 patients, an average of 8,008 patients per year (for comparison, in 2005, before microbiology enhancements, 2,340 patients had blood cultured at the provincial hospital). Median age of patients was 44 years (range 23–73 years). A pathogen was identified in 4,097 (7.2%) patients and *S. suis* in 38 (0.07%). Of the 38 *S. suis* cases, two occurred in 2006, one in 2007, two in 2008, three in 2009, eight in 2010, five in 2011, and 17 in 2012. Fifty-five percent of cases were identified during April–June (Figure). The annual crude incidence ranged from 0.1 to 2.2 cases per 100,000 population; incidence was highest in 2012 (Table 1). Of persons ≥20 years of age (all 38 *S. suis* patients), incidence was highest in 2012 (3.2 cases/100,000 population [range 0.2–3.2/100,000]).

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Table 1. Incidence of *Streptococcus suis*, Nakhon Phanom Province, Thailand, 2006–2012

	Nakhon Phanom			Thailand		
		Active surveillance			Passive surveillance	
Year	Population, all ages	No. cases	Crude incidence*	Population, all ages†	No. cases‡	Crude incidence*
1997				55,747,667	1	0.002
1998				55,747,667	0	0.000
1999				55,747,667	1	0.002
2000				55,747,667	1	0.002
2001				56,305,980	3	0.005
2002				56,840,337	1	0.002
2003				57,345,943	1	0.002
2004				57,830,569	1	0.002
2005				58,319,021	10	0.017
2006	734,000	2	0.27	58,755,907	41	0.070
2007	738,184	1	0.14	59,199,510	90	0.152
2008	742,500	2	0.27	59,626,014	106	0.178
2009	746,655	3	0.40	60,037,264	229	0.381
2010	751,251	8	1.06	60,435,937	207	0.343
2011	754,931	5	0.66			
2012	758,388	17	2.24			

*Per 100,000 persons. Blank cells indicate that data were not reported.
†1997–1999 is assumed to be the same as 2000.
‡In 2010, five cases were in persons from Nakhon Phanom; for all other years, 0 cases occurred.

Within 24 hours after hospital admission, all patients were treated with ceftriaxone. In 12 (32%) patients, permanant deafness developed; all had reported hearing loss at admission. Thirty-five (92%) patients had exposure to pigs or pork in the 7 days before illness onset: 10 (26%), all women, reported preparing pork with their bare hands for consumption and eating undercooked pork, 12 (32%) reported eating both undercooked/uncooked pork and clotted pig blood, and 13 (34%) reported slaughtering pigs for their own consumption. Thirteen patients who reported slaughtering pigs also ate pork but stated that they could not recall how the meat was prepared because of having also consumed alcohol. Seven (18%) patients had acquired pigs in poor health from commercial farms at reduced prices or no cost. Patients resided in 18 (19%) of the 96 subdistricts within Nakhon Phanom, and 10 (26%) patients resided in the same subdistrict. Two clusters of cases occurred in 2012, in which 2 and 3 persons ate raw pork and drank alcohol together. All patients reported no prior knowledge of *S.* suis infection, its symptoms, or ways to prevent infection.

Of the 24 patients with meningitis, 21 (88%) had leukocytosis, 4 (17%) had thrombocytopenia, and 2 (8%) had thrombocytosis. Six of the 24 patients with meningitis had a cerebrospinal fluid (CSF) culture; 1 was positive for *S. suis*. Of the 10 patients with septicemia, 5 had leukocytosis, 1 had leukopenia, 3 had normal leukocyte counts; for 1, leukocyte count was unavailable. Thirty (79%) isolates were initially reported as *Streptococcus* group D nonenterococci by the hospital laboratory; the remaining 8 were reported as other streptococcal groups or species (Table 2). The National Institute of Health reference laboratory identified *S. suis* in 38 patients; all isolates were serotype 2 (PCR and coagglutination results were all concordant). All isolates tested for antimicrobial resistance by disk diffusion (Kirby-Bauer) were susceptible to penicillin (37 isolates) and ceftriaxone (11 isolates). Time from patient blood collection to final pathogen report to the clinician was 30–45 days.

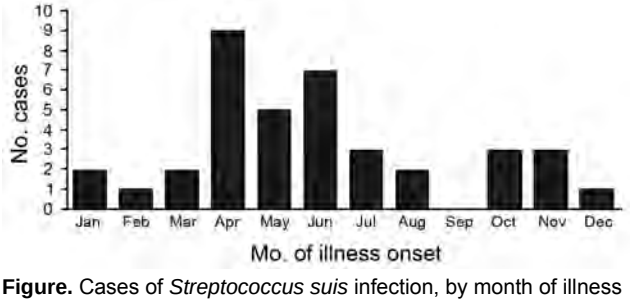

Figure. Cases of *Streptococcus suis* infection, by month of illness onset, Nakhon Phanom Province, Thailand, 2006–2012.

Conclusions

S. suis infection is common in northern Thailand. Here we report laboratory-confirmed cases and incidence in Nakhon Phanom, a northeastern province. Few other studies have reported incidence. The Netherlands reported the most *S. suis* infections in the West (*2*) with an estimated annual incidence of *S. suis* infection of 0.002 cases per 100,000 persons (*11*), and the incidence in northern Thailand was 6.2 cases per 100,000 persons (*12*). Active surveillance suggests that *S. suis* infection might be more common in this region than previously realized (e.g., in 2010, the incidence in Nakhon Phanom was 1.6-fold higher in active than passive reporting; for other years it was greater). During 2006–2012, a total of 45% (17/38) of *S. suis* infection were detected in 2012, including 2 clusters.

Although we did not have a control group with which to compare exposures, our findings are consistent with studies performed in northern Thailand that highlight pork/pig exposure, combined with alcohol use, as a risk factor

Table 2. Characteristics of 38 patients for whom blood culture confirmed *Streptococcus suis* infection, Nakhon Phanom Province, Thailand, 2006–2012

Characteristic	Result of analysis or culture
Male sex, no. (%)	28 (74)
Median age, y (range)	50 (23–73)
Heavy alcohol use, no. (%)*	20 (53)
Current smoker, i.e., smoked daily, no. (%)	21 (55)
Underlying chronic disease, no. (%)	12 (32)
Hypertension	4 (33)
Diabetes	3 (25)
Alcoholism†	3 (25)
Heart disease	1 (0.8)
Gout	1 (0.8)
Occupation: farmer, no. (%)‡	33 (87)
Consumption of/contact with pig or pork product, no. (%)	35 (92)
Days from pork/prok contact to illness onset, median (range)	2 (1–7)
Days from illness onset to hospital admission, median (range)	2 (0–5)
Clinical features	
Meningitis, no. (%)	24 (63)
Septicemia, no. (%)	10 (26)
Arthritis, no. (%)	4 (11)
Laboratory investigation	
Complete blood count, n = 37	
Leukocytosis, >10,000 cells/μL, no. (%)	28 (76)
Leukopenia,<5,000 cells/μL, no. (%)	1 (3)
Thrombocytosis, >450,000 cells/μL, no. (%)	2 (5)
Thrombocytopenia, <150,000 cells/μL, no. (%)	7 (19)
CSF examination,§ n = 12	
Protein, mean ± SD, %	580 ± 421
Glucose, mean ± SD, mg/μL	23 ± 19
Leukocyte count, mean ± SD, cells/μL	2,210 ± 1,580
Polymorphonuclear neutrophils, mean ± SD, %	54 ± 28
Blood culture result reported by hospital laboratory¶	
Streptococcus group D, non-enterococci, no. (%)	30 (79)
Streptococcus group D, no. (%)	2 (5)
Streptococcus pyogenes, no. (%)	1 (3)
Group A β-hemolytic *Streptococcus*, no. (%)	1 (3)
β-Hemolytic *Streptococcus*, no. (%)	1 (3)
Streptococcus not group A,B,D, no. (%)	1 (3)
Enterococcus spp., no. (%)	1 (3)
Streptococcus spp., no. (%)	1 (3)
CSF culture results reported by hospital laboratory, n = 6	
Streptococcus group D, nonenterococci, no. (%)	5 (83)
Streptococcus suis, no. (%)	1 (17)
Days hospitalized, median (range)#	7 (3–19)
Death, no. (%)	0
Permanent deafness, no. (%)**	12 (32)

*Defined as self-reported drinking of >2 alcoholic beverages/day or >14 drinks/week for men and >1 alcoholic beverage/day or >7 drinks/week for women.
†In 1, cirrhosis also was diagnosed.
‡Three raised livestock, and 30 harvested crops (rice).
§Reference values: protein, 15–45 mg/μL; glucose, 50–80 mg/μL; leukocytes, 0–5 cells/μL; polymorphonuclear neutrophils, 0%.
¶All 38 blood culture results were confirmed to be S. suis.
#Excludes 2 patients who experienced septic shock and were transferred to the regional hospital (in another province) on the first day of admission.
**Ten patients had bilateral deafness, of whom 2 with deafness reported chronic ataxia; all 12 had meningitis.

(*13*). Unlike in cases reported in other studies (*12*), no patients reported here died. Patients were treated promptly with ceftriaxone on the first day of admission, which is standard empiric management of suspected sepsis or meningitis in these hospitals. Permanent hearing loss was common, and deafness is usually permanent when it occurs before treatment (*14*).

Our data have several limitations. Blood cultures were performed at clinician discretion and not necessarily for all patients with possible sepsis or meningitis, possibly resulting in missed cases or biasing our study toward the more clinically apparent or severe cases. Blood volume might have been too low for adequate pathogen yield. Only 6 patients had CSF cultures, and most blood and CSF cultures occurred after start of antimicrobial therapy. Therefore, meningitis patients with negative blood cultures might have been missed. Furthermore, because comprehensive examinations were not performed on patients after discharge, neurologic or cognitivie sequalae might have been missed.

Because most hospital laboratories in Thailand are not able to confirm *S. suis*, the infection might be misdiagnosed (*14*). Clinicians in high-risk areas, or who see patients with

recent travel to high-risk areas, should have a low index of suspicion for *S. suis* infection among patients presenting with meningitis or sepsis and recent pig/pork exposure (*15*). Improving the capacity of local laboratories to identify *S. suis* will aid clinical management and facilitate outbreak detection and response. Rapid identification enables faster epidemiologic investigation and swift initiation of control measures (*2*).

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Exposure-Based Screening for Nipah Virus Encephalitis, Bangladesh

Hossain M.S. Sazzad, Stephen P. Luby, Ute Ströher, Peter Daszak, Sharmin Sultana, Sayma Afroj, Mahmudur Rahman, Emily S. Gurley

We measured the performance of exposure screening questions to identify Nipah virus encephalitis in hospitalized encephalitis patients during the 2012–13 Nipah virus season in Bangladesh. The sensitivity (93%), specificity (82%), positive predictive value (37%), and negative predictive value (99%) results suggested that screening questions could more quickly identify persons with Nipah virus encephalitis.

Nipah virus (NiV) is a bat-borne paramyxovirus that causes encephalitis in humans and can be transmitted from person to person, posing a global pandemic threat (*1*–*4*). Because of the high costs of laboratory-based surveillance systems, maintaining surveillance for highly fatal but rare diseases, such as NiV encephalitis, is difficult for low-income countries, such as Bangladesh. In Bangladesh, NiV encephalitis outbreaks with a >70% case-fatality rate have occurred almost every year since 2001 during December–April (*3*–*5*). Where NiV encephalitis outbreaks had been repeatedly identified, the Institute for Epidemiology Disease Control and Research of the Government of Bangladesh, in collaboration with icddr,b, started hospital-based encephalitis surveillance in February 2006.

Before illness onset, nearly every NiV encephalitis case-patient identified in Bangladesh was exposed either to raw or fermented date palm sap or to another NiV encephalitis case-patient (*3*–*6*). Bangladeshis collect and drink raw date palm sap fresh during December–April (*3*–*5*). *Pteropus* fruit bats come in contact with date palm sap by landing on the sap stream and contaminate sap with saliva and/or urine (*7*,*8*). NiV RNA has been identified in human blood, urine, respiratory secretions, and saliva (*4*,*9*). Epidemiologic studies indicate that respiratory secretions and saliva are the most likely vehicles of NiV transmission from patients to caregivers (*3*,*4*). Therefore, implementation of standard, contact, and droplet precautions (*10*) could reduce NiV transmission in hospitals. However, hospitals with few resources for infection control are unable to implement standard, contact, and droplet precautions for all patients with encephalitis (*11*), which places caregivers at risk for person-to-person transmission of NiV (*4*,*12*).

Because no rapid diagnostic tests for NiV infection are currently available, surveillance for NiV encephalitis in Bangladesh relies on a central laboratory in the capital (Dhaka) to confirm diagnosis several days or weeks after sample collection. Earlier identification of NiV encephalitis cases would enable targeted infection control efforts to reduce the number of persons exposed to NiV-infected patients and thereby reduce subsequent secondary transmission of NiV from person to person. The objective of this study was to compare the performance of screening questions about recent exposure to date palm sap or other encephalitis patients with serologic testing results to more quickly identify NiV encephalitis cases in our surveillance hospitals.

The Study

Since 2006, surveillance physicians have listed and collected blood from patients with encephalitis, defined as fever or history of fever with axillary temperature >38.5°C (101.3°F) and altered mental status, new onset of seizures, or new neurologic deficit—in patients admitted to 3 Nipah surveillance hospitals: Rajshahi, Rangpur, and Faridpur Medical College hospitals. The Institute for Epidemiology Disease Control and Research and US Centers for Disease Control and Prevention tested serum with an IgM-capture enzyme immunoassay to detect NiV IgM, and we defined laboratory-confirmed NiV encephalitis as NiV IgM in serum.

During December 2012–March 2013, surveillance physicians interviewed accompanying caregivers of all hospitalized patients whose illness met the encephalitis case definition on admission in the inpatient ward. Study physicians asked about patients' consumption of raw or fermented date palm sap and contact with other persons with fever and altered mental status in the month before illness onset; if caregivers were unaware of the patient's exposures, study physicians asked them phone the patient's friends and colleagues about exposures. Exposures were recorded in surveillance log books. Hospital physicians used personal protection equipment and provided it

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Table 1. Proportion of encephalitis patients with history of reported exposures to drinking date palm sap or contact with patients with encephalitis by Nipah serodiagnosis in 3 surveillance hospitals, Bangladesh, 2012–13 Nipah virus season

	Overall, Dec–Mar, no. (%)			Peak incidence during Jan–Feb, no. (%)		
Reported risk factor	IgM positive, n = 17	IgM negative, n = 311	Total, n = 328	IgM positive, n = 15	IgM negative, n = 132	Total, n = 147
History of drinking raw date palm sap	9 (47)	45 (14)	54 (16)	9 (60)	24 (18)	33 (22)
History of drinking fermented date palm sap	2 (11)	0	2 (1)	2 (13)	0	2 (1)
History of contact with other encephalitis patients	3 (18)	0	3 (1)	3 (20)	0	3 (2)
No history of drinking raw or fermented date palm sap or contact with encephalitis patients	3 (18)	266 (86)	269 (82)	1 (7)	108 (5)	109 (47)

to caregivers of each patient with encephalitis and a history of these exposures.

As part of subsequent epidemiologic studies, we also conducted detailed case investigations at each NiV encephalitis case-patient's household. We interviewed surviving patients directly, or appropriate proxies among family, friends and relatives for patients who died, about their exposures to encephalitis patients or to fresh or fermented date palm sap before illness.

We calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the screening questions asked on admissions to hospitals by comparing with the NiV IgM results. We repeated the calculations for patients hospitalized during January and February, when the prevalence of NiV encephalitis is highest. We compared the answers provided by caregivers during patient hospitalization with those provided during interviews in the community as part of our epidemiologic studies. icddr,b's Ethical Review Committee reviewed and approved the protocol for NiV surveillance and case investigation.

Surveillance physicians identified 360 patients with encephalitis during December 2012–March 2013. They collected and tested blood samples from 328 (91%) patients for NiV IgM. Seventeen (5%) had NiV IgM (Table 1), of whom 15 (88%) NiV encephalitis case-patients were identified during January and February 2013. Of the 17 confirmed case-patients, family caregivers of 14 reported either a history of drinking raw or fermented date palm sap or contact with other persons with fever and altered mental status in the month before illness onset. Therefore, the sensitivity of the screening questions was 82%, specificity was 86%, PPV was 24% and NPV was 99% (Table 2). The sensitivity during January–February was 93%, specificity was 82%, PPV was 37%, and NPV was 99%.

At admission, 3 (18%) NiV encephalitis case-patients had no reported history of drinking raw or fermented date palm sap or of contact with persons who had encephalitis (Table 1). However, during the epidemiologic investigations in the community, family members of 2 case-patients reported that the patients drank fermented date palm sap in the month before illness onset. Of the 14 NiV encephalitis case-patients who, at admission, had reported 1 of the risk exposures, results were consistent with exposures reported during the epidemiologic investigation.

Conclusions

Screening patients with possible encephalitis at the time they seek hospital care regarding recent exposure to date palm sap and to other patients with encephalitis demonstrated high sensitivity and specificity for detecting NiV encephalitis, particularly during peak months of NiV encephalitis incidence. The high NPV of the screening questions suggests that focusing infection control efforts toward patients with these exposures is an efficient use of scarce resources to prevent transmission. Although three fourths of encephalitis patients had reported histories of exposure, they possibly could have had other infections, including other bat-borne viruses, that were transmitted through similar routes or could have lacked NiV IgM, despite having NiV infection (*13*). Alternatively, recent consumption of date palm sap by these patients might have been purely coincidental because this practice is common in Bangladesh during this season, but Nipah infection is rare.

For 2 NiV encephalitis case-patients, caregivers did not report a history of drinking fermented date palm sap during hospital interview, but this behavior was reported in later community investigations. Because 90% of Bangladeshis are Muslim, and consumption of alcohol is prohibited

Table 2. Comparison of results of screening for exposure to* and results of serologic testing for Nipah virus encephalitis among patients with encephalitis at 3 surveillance hospitals, Bangladesh, December 2012–March 2013

Months	Prevalence (95% CI), %	Sensitivity (95% CI), %	Specificity (95% CI), %	Positive predictive value (95% CI), %	Negative predictive value (95% CI), %
Dec 2012–Mar 2013	5 (3–8)	82 (57–96)	86 (81–89)	24 (14–37)	99 (97–100)
Jan–Feb 2013	10 (6–16)	93 (68–100)	82 (74–88)	37 (22–54)	99 (95–100)

*Drinking raw or fermented date palm sap or having contact with encephalitis patients in month before illness onset.

by Islam (*14*) and illegal in Bangladesh, patients might be reluctant to report drinking traditional liquor made of fermented date palm sap. Therefore, caregivers should be asked about socially stigmatized behaviors privately and confidentially to increase the odds that these stigmatized behaviors are reported.

Exposure-based screening can detect patients at high risk for NiV encephalitis in low-income, resource-constrained settings, such as Bangladesh. We deployed screening questions on admission to inpatient wards but screening earlier, at triage in emergency wards, could further reduce risk. Surveillance for other diseases with well-described exposures that put healthcare workers at risk, such as Ebola virus infection, and where laboratory diagnosis is limited or delayed could also deploy this approach.

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Close Relationship between West Nile Virus from Turkey and Lineage 1 Strain from Central African Republic

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We sequenced West Nile viruses (WNVs) from Turkey and found close relationships to WNV lineage 1 strain ArB310/67 from the Central African Republic, distinct from other WNVs circulating in the Mediterranean Basin, eastern Europe, and the Middle East. These findings suggest independent introductions of WNV strains from Africa to the Middle East.

West Nile virus (WNV; family *Flaviviridae*, genus *Flavivirus*) (*1*) is a mosquito-borne avian virus that can cause febrile illness and potentially life-threatening neuroinvasive disease in mammalian hosts, predominantly in humans and horses (*2*). WNV is widely distributed in Africa; the Middle East; central, southern and eastern Europe; southwestern Russia; southwestern Asia and Australia (*3*); and North, Central, and South America (*4*,*5*).

Initial evidence for WNV circulation in Turkey was provided by serosurveillance reports (*6*). Since 2009, sporadic human and equine cases and limited disease outbreaks have demonstrated the occurrence of symptomatic infections comprising self-limiting febrile diseases as well as central nervous system infections resulting in occasional deaths (*6*–*8*). Current data reveal widespread WNV circulation in Turkey, including virus detection in competent mosquito species (*8*–*10*). To determine the lineage and relationships of WNVs circulating in Turkey, we analyzed the complete genome sequence of the initially reported WNV isolate from Turkey and several partial sequences from isolates detected from various sources and locations in this country during 2011–2013.

The Study

WNV strain T2 was isolated from a horse originating from Eskisehir Province (39°24′N–31°02′E), Central Anatolia, Turkey; the horse had febrile illness beginning on January 18, 2011, and neurologic signs subsequently developed (*8*). The complete genome sequence was amplified by using continuous reverse transcription PCR assays and WNV lineage 1–specific primers as previously described (*11*). Amplification products were directly sequenced in both directions (Microsynth, Balgach, Switzerland) using the amplification primers. The resulting overlapping partial sequences were verified by BLAST search (http://www.ncbi.nlm.nih.gov/blast/) and compiled to 1 continuous sequence. Three regions, not covered by the reactions, were amplified with novel forward (f) and reverse (r) primers: 4275f, 5′-GACTATCGCGGGGCTCATGT-3′; 4686r, 5′-GCCCGCTCCTGCTTGATAAC-3′; 4940f, 5′-GGGCCGTGACTTTGGACTTC-3′; 5290r, 5′-TGGGCAGTCCTCTCAGTGCT-3′; 7968f, 5′-TGAAGAGCCCCAACTAGTGC-3′; and 8277r, 5′-CCGTGAGAGTGGGTTTCTGA-3′. (Region numbers refer to nucleotide positions in WNV strain HNY1999, GenBank accession no. AF202541). These regions were then sequenced, and the sequences were compiled with the previously determined sequences. The complete genome of WNV strain T2 was compared with genomes of 32 other WNV strains, and phylogenetic analysis was performed. For sequence handling and phylogenetic analyses, we used CLC Main Workbench version 5.5 (CLCBio, Aarhus, Denmark) and MEGA version 6.0.5 (http://www.megasoftware.net/).

The T2 genome consists of 11,026 nt (GenBank accession no. KJ958922); the 5′ and 3′ untranslated regions comprise 93 (nt 1–93) and 630 (nt 10396–11026) bases, respectively. The putative open reading frame (nt 93–10395) was translated to a 3,433-aa polypeptide that encodes viral capsid (C, positions 1–105); C-anchor peptide (ER, positions 106–123); premembrane protein (prM, positions 124–290); envelope protein (E, positions 291–791); and nonstructural (NS) proteins NS1 (positions 792–1143), NS2A (positions 1144–1374), NS2B (positions 1375–1505), NS3 (positions 1506–2124), NS4A (positions 2125–2250), peptide 2k (positions 2251–2273), NS4B (positions 2274–2528), and NS5 (positions 2529–3433). Comparison of the complete nucleotide sequence with those of several other lineage 1 WNV strains revealed 0.7% (strain ArB310/67) to 20.6% (strain G16146, clade 1c) divergence (data not shown). Phylogenetic analyses of the nucleotide and amino acid sequences demonstrated, supported by high bootstrap values, that strain T2 clusters within WNV lineage 1 clade 1a viruses, forming a distinct cluster together with WNV strain ArB310/67, which was isolated in 1967 in the Central African Republic (Figure 1). Similar or identical topologies were observed in maximum-likelihood and UPGMA trees (data not shown).

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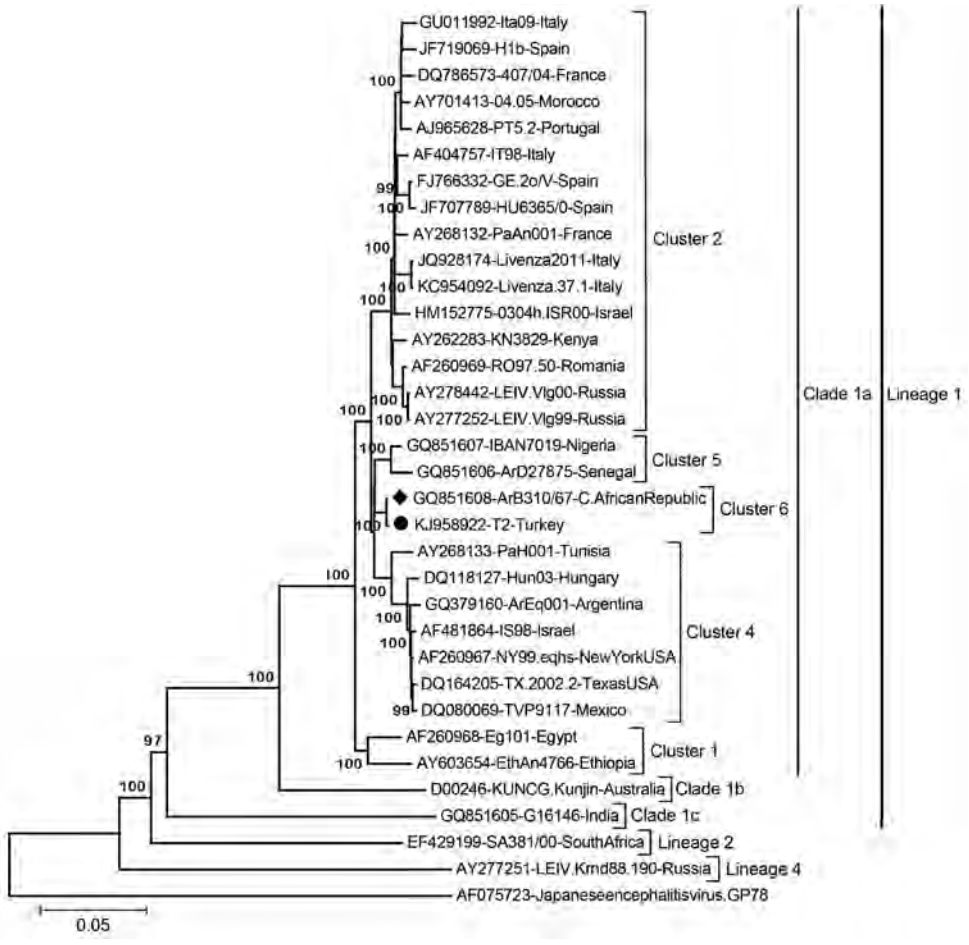

Figure 1. Neighbor-joining phylogenetic tree constructed on the basis of the complete genomic nucleotide sequences of selected West Nile virus (WNV) strains, including isolate T2 from Turkey (black dot) and the closely related strain ArB310/67 from the Central African Republic (black diamond). Major WNV lineages, clades, and clusters are indicated, except for the local cluster 3 (*12*). Bootstrap values of major branches are given for 1,000 replicates. Virus strains are described by GenBank accession number, name, and country of isolation. Japanese encephalitis virus strain GP78 served as outgroup. Scale bar indicates substitutions per site.

To evaluate whether T2 is the main WNV strain circulating in Turkey or whether divergent strains are co-circulating, we investigated another 37 WNV isolates, detected during 2011–2013 from different geographic regions of Turkey and from human, equine, and mosquito samples. From these samples, only partial envelope (E) gene sequences were available; these sequences consisted of 212–256 nt and corresponded to amino acid positions 174–255 in the T2/NY99 WNV polyprotein and have been deposited in GenBank (accession nos. JN828805, JX310862, JX310863, KC290932–KC290942, KC466019–KC466021, KF437832, and KJ433822–KJ433840). These 37 partial E gene sequences exhibited 98.8%–100% nucleotide identities to each other, regardless of collection date, geographic area, and source of isolation (data not shown). Neighbor-joining phylogenetic analysis of a common 183-nt stretch of these isolates revealed that all but 1 clustered together with the T2 and ArB310/67 strains (Figure 2). Maximum-likelihood and UPGMA analyses yielded similar results (data not shown). One isolate (GenBank accession no. JN828805), identified from a horse in February 2011 in Central Anatolia, clustered in a sister subclade with 2 other older WNV strains from Africa (strain IBAN7019, isolated in 1965 in Nigeria, and strain ArD27875, isolated in 1979 in Senegal).

WNV lineage 1 strains have a widespread geographic distribution throughout Africa, Europe, the Middle East, and North America, and have been divided into 3 clades, of which clade 1a contains the largest number of strains; these strains can be further grouped into 6 distinct clusters (*12*). The phylogenetic analyses in our study confirm that the various clusters are well separated and that the T2 strain and 36 of 37 other WNV strains from Turkey are located in cluster 6, together with strain ArB310/67 from the Central African Republic (Figures 1, 2). However, subclustering of the sequences in cluster 6 (Figure 2) is statistically poorly supported because of the relatively short sequences analyzed and the few common nucleotide substitutions.

Amino acid substitutions that delineate WNV strains belonging to cluster 1 (NS4B-S11N), cluster 3 (NS2A-A224T), cluster 4 (E-T126I, NS4A-V85A), and the eastern European subtype of cluster 2 (NS1-L206F, NS2B-A103V, NS3-T249P and NS5-T898I) were not found in the T2 WNV strain from Turkey (*12*). Likewise, substitutions that have become established in the North America WNV populations (E-K291R, NS4A-A85T, and NS5-K318R)

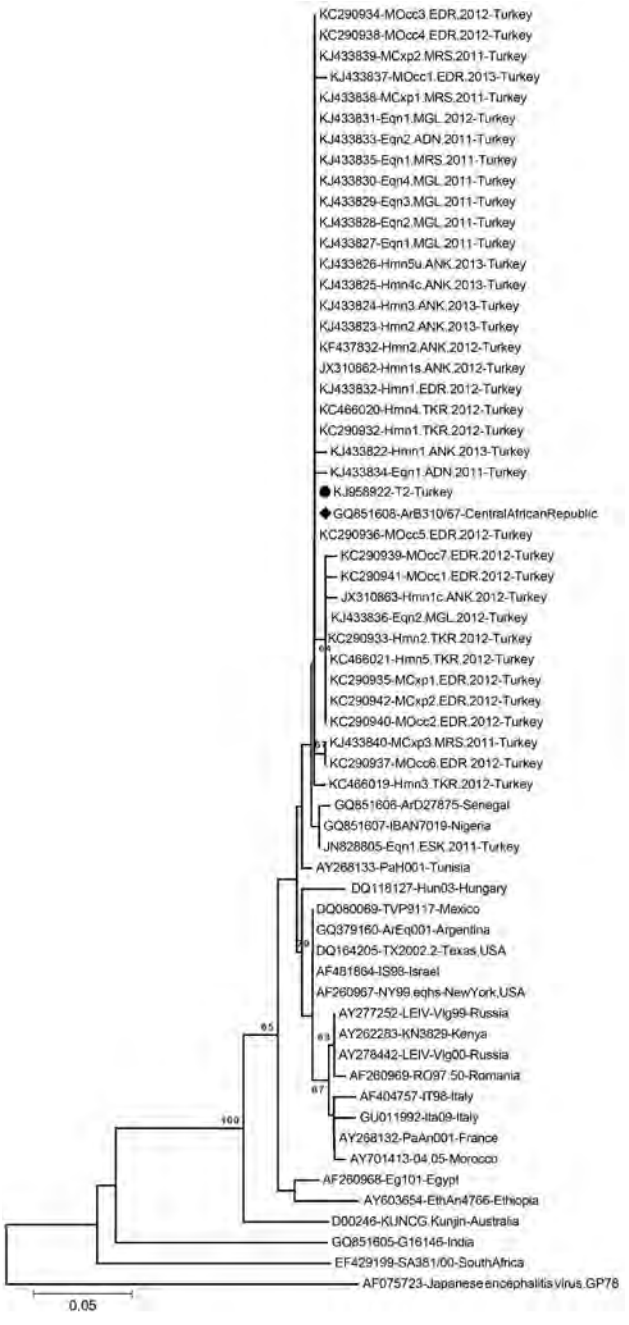

Figure 2. Neighbor-joining phylogenetic tree constructed on the basis of the West Nile virus (WNV) partial envelope gene nucleotide sequences (183 nt) of 38 WNV strains from Turkey and selected global strains. Black dot indicates WNV isolate T2 from Turkey, and black diamond indicates the closely related strain ArB310/67 from the Central African Republic. Bootstrap values of major branches are given for 1,000 replicates. GenBank accession number, name, and country of isolation are given for global strains. Local sequences are indicated by GenBank accession number, host, location, and year. ADN, Adana Province; ANK, Ankara Province; Cxp, *Culex pipiens*; EDR, Edirne Province; Eqn, equine; ESK, Eskisehir Province; Hmn, human; M, mosquito, MGL, Mugla Province; MRS, Mersin Province; Occ, *Ochleratatus caspius*; TKR, Tekirdag Province. Scale bar indicates substitutions per site.

were absent from this strain. However, the NS1-A70S substitution noted in cluster 2 viruses was observed as A70V in strain T2. Furthermore, NS3-P249T and NS5-V258A substitutions, reported in several strains of the Mediterranean subtype (*12*), were detected in the T2 strain. These findings suggest that the T2 strain evolved independently from other Mediterranean strains from a common ancestor of African origin.

The T2 strain retained the original WNV envelope protein glycosylation motif (E154–156, NYS) and lacked substitutions associated with neurovirulence attenuation (NS4b-C102S, NS2A-A30P) and increased virulence for crows and humans (NS3-T249P) (*12*,*13*). However, the E-V159A change in the WN02 genotype that was associated with a shorter extrinsic incubation period in *Culex* spp. mosquitoes (*14*) was noted as V159F in this isolate. A few other amino acid variations with unknown implications were also observed (data not shown).

Conclusions

The evaluation of the complete nucleotide and amino acid sequences of the T2 WNV isolate from Turkey revealed a close genetic relationship with strain ArB310/67, isolated in 1967 in the Central African Republic. These 2 virus strains form a distinct genetic cluster within WNV lineage 1a strains. This observation is supported by analysis of partial E gene sequences detected in temporally and spatially separated sources in Turkey. The investigated circulating WNV strains from Turkey proved to be genetically distinct from viruses circulating in eastern Europe, the Mediterranean region, and the Middle East. However, several amino acid substitutions detected in viruses from throughout the Mediterranean region were identified in strain T2, including those that might affect virus replication in vector mosquitoes. A recent report on a lineage 2 WNV obtained from a human patient in Iran in 2009 also revealed 99% nucleotide identity to a WNV strain collected in the Central African Republic (*15*). These findings suggest independent introductions of WNV strains of African origin to the Middle East, potentially by migratory birds, and the accumulation of adaptive changes during circulation.

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Zoonotic Transmission of Toxigenic *Corynebacterium ulcerans* Strain, Germany, 2012

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Severe necrotizing fasciitis was diagnosed in a 53-year-old man in Germany in 2012. Toxigenic *Corynebacterium ulcerans* was grown from a wound swab sample. One of the patient´s 2 dogs was found to harbor a toxigenic *C. ulcerans* strain. Results of next generation sequencing of both isolates supported recent zoonotic transmission of this bacterial pathogen.

Toxigenic *Corynebacterium* spp. *C. diphtheriae*, *C. ulcerans*, and rarely, *C. pseudotuberculosis* produce diphtheria toxin (DT) and thus cause respiratory and cutaneous diphtheria. DT is encoded by the phage-located *tox* gene. During the past decade, diphtheria-like infections with toxigenic *C. ulcerans* have outnumbered those caused by toxigenic *C. diphtheriae* in many industrialized countries (*1*). *C. ulcerans* has increasingly been isolated from domestic animals such as pet dogs and cats (*1*–*5*). Isolation of an undistinguishable toxigenic *C. ulcerans* strain from an animal and its owner has been documented for 2 dogs (*2*,*3*), 2 cats (*4*,*5*), and 1 pig (*6*) and their respective owners. Strain comparison was achieved in these cases by ribotyping alone (*2*,*3*), ribotyping in combination with multilocus sequence typing (*4*,*6*), or pulsed-field gel electrophoresis analysis (*5*). We report the first use of next generation sequencing (NGS) for proving zoonotic transmission of a toxigenic *C. ulcerans* strain between a pet dog and his human owner.

The Study

In October 2012, a 53-year-old man in Baden-Wuerttemberg, Germany, whose only known underlying condition was chronic venous insufficiency, sought treatment from a private physician, who diagnosed severe necrotizing fasciitis in the calves of both legs. The patient reported no trauma, and gave no history of recent travel abroad or contact with livestock animals. The patient lived alone with his 2 dogs and had no other close personal contacts. The patient´s vaccination status against diphtheria was unknown. A swab sample from the wound on his right leg grew *Staphylococcus aureus*, *Bacteroides* spp., and *Corynebacterium ulcerans*, which were identified by biochemical differentiation (API Coryne code 0111326), including a positive O129 sensitivity test result, *rpoB* sequencing, and a score of 2.463 from Matrix-assisted laser desorption/ionisation time-of-flight analysis (MALDI Biotyper; Bruker Daltonics, Bremen, Germany). The isolate was identified as toxigenic by a positive real-time PCR for the DT-encoding *tox* gene (*7*) and a positive cytotoxicity assay by using Vero cells (toxigenic titer: 1.8; toxigenic titer of the highly toxigenic *C. diphtheriae* control NCTC 10648: 1:2000) as reported by Sing et al (*8*). However, a modified Elek test yielded a negative result (*8*). The patient was isolated at home, where he was treated with penicillin and clindamycin for 14 days and received surgical wound debridement several times over a period of 4 weeks, after which he recovered completely. The patient´s isolation at home was discontinued after 2 wound swab samples obtained 2 weeks after treatment ended showed no growth of *C. ulcerans*.

The local health authority started a comprehensive source investigation. No close human contacts could be identified. Because of known zoonotic transmission of *C. ulcerans* to humans, however, nasal and pharyngeal swab samples from the patient´s 2 asymptomatic pet dogs were obtained by the local veterinary authority. Cultures of nasal and pharyngeal swab samples from 1 of his 2 dogs grew toxigenic *C. ulcerans* and showed an API Coryne code identical to that of the human isolate. The other dog´s cultures grew several species of normal canine bacterial flora. Antimicrobial susceptibility of the isolates was tested on Mueller-Hinton blood agar (supplemented with 5% sheep blood) after overnight incubation at 37°C and 5% CO_2. In the absence of standardized breakpoints for *C. ulcerans*, antibiotic susceptibility was determined by using the CLSI

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criteria for broth microbouillon dilution susceptibility testing for *Corynebacterium* species (*9*).

Both the human and the dog *C. ulcerans* strains were susceptible to amoxicillin, benzyl penicillin, ceftriaxone, erythromycin, ciprofloxacin, vancomycin, linezolid, and tetracycline, and showed intermediate susceptibility to clindamycin. Although currently no definite recommendations exist regarding antibiotic treatment of animals infected or colonized with *C. ulcerans* for preventing possible animal-to-human transmission of diphtheria-associated *Corynebacterium* spp. infection, antibiotic treatment was discussed as an option, but refused by the dog owner.

Because sequencing of *rpoB* and *tox* yielded 100% homology between the strains isolated from human and dog samples and multilocus sequence typing (*4*) suggested the clonal identity of both isolates, we aimed to confirm these findings by NGS. For a detailed genomic characterization of both isolates, we performed genome-wide resequencing on an Illumina MiSeq instrument (Illumina, Eindhoven, the Netherlands). Using Burrows-Wheeler Aligner software (SourceForge.net, Dice Holdings, New York, New York, USA), we mapped readings of both isolates to the reference genome *C. ulcerans 809* (*10*) and used VarScan (http://varscan.sourceforge.net/) for single nucleotide polymorphism (SNPs) verification. SNPs found in both isolates were discarded from the SNP list (≈20,000 SNPs compared to *C. ulcerans 809*). Next, we discarded the SNPs that were only found in 1 of the isolates because of technically missing sequence coverage in the other isolate data. We found that the 2 isolates did not differ by any SNPs throughout the 2.5 MB genome, clearly showing the clonal identity of both isolates. The absence of SNPs between the 2 isolates suggests a recent zoonotic transmission, because we would expect the isolates to accumulate SNPs if the transmission happened long before the strains were isolated (*11*). Furthermore, we did not detect any additional gene acquisition or genomic reordering, such as inversion or transposition in 1 of the 2 isolates but not the other. Additionally, we analyzed DT loci of both isolates and found that the DT gene is encoded by a toxigenic prophage, which is almost identical to the toxigenic phage of *C. ulcerans 0102* isolate as shown by Sekizuka et al. (*12*) in Japan.

Conclusions

Our finding of a transmission of toxigenic *C. ulcerans* between a dog and his human owner proven by NGS underlines both the usefulness of this novel technology in a zoonotic setting of potentially toxigenic *Corynebacterium* spp. and the zoonotic potential of this organism. Previously, the proof of zoonotic transmission of *C. ulcerans* mainly had to rely on less reproducible or standardized typing methods which are more dependent on subjective interpretation, e.g., ribotyping (*2–4*,*6*) or pulsed-field gel electrophoresis (*5*), or on the epidemiologic circumstances in the case of deep cutaneous diphtheria manifestation caused by toxigenic *C. ulcerans* that was obviously transmitted by a cat bite (*13*). To our knowledge, the only previous use of NGS for proving zoonotic transmission of a bacterial pathogen by analyzing epidemiologically linked human and animal isolates was reported in a suspected MRSA outbreak investigation involving 2 farms where 2 separate outbreaks with sheep-to-human and cow-to-human transmission were detected by using NGS (*14*).

We found the toxigenic strain involved in the case-patient´s skin ulceration to be *C. ulcerans*–negative using a modified Elek test, but positive in a cytotoxicity assay using Vero cells. Similar findings have been reported previously for toxigenic *C. ulcerans*, suggesting a higher sensitivity of cytotoxicity assays than that seen with the Elek test (*15*). Because DT production by toxigenic *C. ulcerans* is reported to be substantially lower than that usually seen in toxigenic *C. diphtheriae* (*8*) as was the case in the study patient, it is difficult to estimate the pathogenic contribution of these low amounts of DT detected only in vitro by using a cytotoxicity assay, but not by immunologic precipitation for this patient in vivo. Furthermore, as described first for *C. diphtheriae*, also nontoxigenic *tox*-bearing strains of *C. ulcerans* exist, and some of them originate in animals (*8*).

In conclusion, the declining costs of NGS and the increasing availability of bioinformatics tools should make this method more available. The wider introduction of this technology in public health laboratories will help in outbreak investigations and support public health authorities in the management of infectious diseases.

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Potential Sexual Transmission of Zika Virus

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In December 2013, during a Zika virus (ZIKV) outbreak in French Polynesia, a patient in Tahiti sought treatment for hematospermia, and ZIKV was isolated from his semen. ZIKV transmission by sexual intercourse has been previously suspected. This observation supports the possibility that ZIKV could be transmitted sexually.

Zika virus (ZIKV) is a mosquitoborne arbovirus in the family *Flaviviridae*, genus *Flavivirus*. It was first isolated in 1947 from a rhesus monkey in the Zika forest of Uganda (*1*). Sporadic human cases were reported from the 1960s in Asia and Africa. The first reported large outbreak occurred in 2007 on Yap Island, Federated States of Micronesia (*2*). The largest known ZIKV outbreak reported started in October 2013 in French Polynesia, South Pacific (*3*), a territory of France comprising 67 inhabited islands; an estimated 28,000 persons (11% of the population) sought medical care for the illness (*4*). The most common symptoms of Zika fever are rash, fever, arthralgia, and conjunctivitis. Most of the patients had mild disease, but severe neurologic complications have been described in other patients in French Polynesia (*5*).

The Study

In early December 2013, during the ZIKV outbreak, a 44-year-old man in Tahiti had symptoms of ZIKV infection: asthenia, low grade fever (temperature from 37.5°C to 38°C) and arthralgia. Symptoms lasted 3 days. Eight weeks later, he described a second episode of symptoms compatible with ZIKV infection: temperature from 37.5°C to 38°C, headache on days 1–3, and wrist arthralgia on days 5–7. The patient did not seek treatment, thus biological samples were not collected during the first 2 periods of illness. The patient fully recovered from the second episode, but 2 weeks later he noted signs of hematospermia and sought treatment. Because the patient had experienced symptoms of ZIKV infection some weeks before, he was referred to our laboratory in the Institut Louis Malardé, Papeete, Tahiti for ZIKV infection diagnostic testing. The medical questionnaire revealed no signs of urinary tract infection, prostatitis, urethritis, or cystitis, and the patient stated that he did not had any recent physical contact with persons who had acute ZIKV infection. We collected blood and semen samples. Direct and macroscopic examinations of the semen confirmed hematospermia. We extracted RNA using the NucliSENS easyMAG system (bioMérieux, Marcy l'Etoile, France) from 200 µL of blood and from 500 µL of semen and urine; both were eluted by 50 µL of elution buffer. We used 5 µL of RNA extracted for amplification. We tested blood and semen RNA extracts using real-time reverse transcription PCR (rRT-PCR) as described using 2 primers/probe amplification sets specific for ZIKV (*3*). The rRT-PCR results were positive for ZIKV in semen and negative in blood, and confirmed by sequencing of the genomic position 858–1138 encompassing the prM/E protein coding regions of ZIKV. The generated sequence (GenBank accession no. KM014700) was identical to those previously reported at the beginning of the ZIKV outbreak (*3*). Three days later, we collected a urine sample, then a second set of blood and semen samples. Semen and urine from this second collection were not found to contain traces of blood by both direct and macroscopic examinations. rRT-PCR detected ZIKV RNA in the semen and urine, but not in the blood sample.

We quantified ZIKV RNA loads using an RNA synthetic transcript standard that covers the region targeted by the 2 primers/probe sets. RNA loads were: 2.9×10^7 copies/mL and 1.1×10^7 copies/mL in the first and second semen samples, respectively, and 3.8×10^3 copies/mL in the urine sample.

We cultured semen and urine as described for dengue virus cultured from urine (*6*). Briefly, 200 µL of each sample diluted in 200 µL of 1% fetal calf serum (FCS) minimum essential medium (MEM) were inoculated onto Vero cells and incubated for 1 h at 37°C; inoculum was then removed and replaced by 1 mL of culture medium. We also inoculated a negative control (200 µL of 1% FCS-MEM) and a positive control (5 µL of a ZIKV-positive serum diluted in 200 µL of 1% FCS-MEM). The cells were then incubated at 37°C in 5% CO_2 for 6 days. The presence of ZIKV in the culture fluids was detected by rRT-PCR as described.

Replicative ZIKV particles were found in the 2 semen samples but none were detected in the urine sample. This finding does not exclude the possibility that ZIKV particles were present in urine. Positive samples were not titered.

Conclusions

The ZIKV natural transmission cycle involves mosquitoes, especially *Aedes* spp. (*7*), but perinatal transmission (*8*) and potential risk for transfusion-transmitted ZIKV infections has also been demonstrated (*9*). Moreover, ZIKV

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transmission by sexual intercourse has been suggested by Foy et al. (*10*), who described a patient who was infected with ZIKV in southeastern Senegal in 2008. After returning to his home in Colorado, United States, he experienced common symptoms of ZIKV infection and symptoms of prostatitis. Four days later, he observed signs of hematospermia, and on the same day, his wife had symptoms of ZIKV infection. Because the wife of the patient had not traveled out of the United States during the previous year and had sexual intercourse with him 1 day after he returned home, transmission by semen was suggested. ZIKV infection of the patient and his wife was confirmed by serologic testing, but the presence of ZIKV in the semen of the patient was not investigated.

Infectious organisms, especially sexually transmitted microorganisms including viruses (human papillomavirus or herpes simplex virus), are known to be etiologic agents of hematospermia (*11*). To our knowledge, before the report of Foy et al. (*10*) and this study, arbovirus infections in humans had not been reported to be associated with hematospermia, and no arboviruses had been isolated from human semen.

We detected a high ZIKV RNA load and replicative ZIKV in semen samples, but ZIKV remained undetectable by rRT-PCR in the blood sample collected at the same time. These results suggest that viral replication may have occurred in the genital tract, but we do not know when this replication started and how long it lasted. The fact that the patient had no common symptoms of ZIKV acute infection concomitantly to hematospermia suggests that the viremic phase occurred upstream, probably during the first or second episode of mild fever, headache, and arthralgia.

The detection of ZIKV in both urine and semen is consistent with the results obtained in a study of effects of Japanese encephalitis virus, another flavivirus, on boars. The virus was isolated from urine and semen of experimentally infected animals, and viremia developed in female boars that artificially inseminated with the infectious semen (*12*).

Flaviviruses have also been detected in urine of persons infected with West Nile virus (WNV). WNV RNA was detected in urine for a longer time and with higher RNA load than in plasma (*13*). WNV antigens were detected in renal tubular epithelial cells, vascular endothelial cells, and macrophages of kidneys from infected hamsters (*14*), suggesting that persistent shedding of WNV in urine was caused by viral replication in renal tissue. Dengue virus (DENV) RNA and DENV nonstructural protein 1 antigen were also detected in urine samples for a longer time than in blood, but infectious DENV has not been isolated in culture. Hirayama et al. concluded that the detection of DENV by rRT-PCR was useful to confirm DENV infections after the viremic phase (*6*). Also, yellow fever virus RNA was isolated from the urine of vaccinated persons (*15*), and Saint Louis encephalitis viral antigens, but not infective virus, have been detected in urine samples from infected patients (*10*).

Our findings support the hypothesis that ZIKV can be transmitted by sexual intercourse. Furthermore, the observation that ZIKV RNA was detectable in urine after viremia clearance in blood suggests that, as found for DENV and WNV infections, urine samples can yield evidence ofZIKV for late diagnosis, but more investigation is needed.

We obtained written informed consent from the patient for publication of this report, and publication of data related to ZIKV infections have been approved by the Ethics Committee of French Polynesia under reference 66/CEPF.

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Cluster of Middle East Respiratory Syndrome Coronavirus Infections in Iran, 2014

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During January 2013–August 2014, a total of 1,800 patients in Iran who had respiratory illness were tested for Middle East respiratory syndrome coronavirus. A cluster of 5 cases occurred in Kerman Province during May–July 2014, but virus transmission routes for some infections were unclear.

Middle East respiratory syndrome coronavirus (MERS-CoV) was initially reported in September 2012 in Saudi Arabia (*1*); the first human infected died of respiratory and renal failure (*2*,*3*). As of July 23, 2014, a total of 837 human cases and 291 deaths had been reported (*4*); all cases were directly or indirectly linked to travel to or residence in the Middle East.

During January 2013–August 2014, a total of 1,800 patients in Iran who had respiratory illness were tested for MERS-CoV. Patients tested during 2013 had been pilgrims to Mecca, Saudi Arabia, during the Hajj; patients tested during 2014 were pilgrims or had been hospitalized for respiratory infections with unknown causes. We report a cluster of 5 cases that occurred in the same hospital in Kerman Province, Iran, during May–July 2014 (Table).

The Cases

Patient 1 was a 52-year-old woman with a history of hypertension who became ill on May 1, 2014, and was admitted to hospital A on May 11 with high fever (temperature >38°C), cough, dyspnea, diarrhea, and anorexia. Her condition deteriorated, and she was transferred to an intensive care unit (ICU). Her condition remained poor, and on May 29, 18 days after her symptoms began, she died of progressive respiratory failure. Patient 1 had not traveled to Saudi Arabia, but she had had close contact with a woman who had influenza-like illness and who had traveled to Saudi Arabia 2 weeks before her symptoms began. This contact of patient 1 is suspected of being the index case-patient, but when throat swab and sputum samples were collected from her, she had no symptoms, and PCR results were negative. A serum sample was not tested because serologic testing for MERS-CoV was not available.

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Patient 2 was the 50-year-old sister of patient 1 and also had a history of hypertension. She became ill on May 11, 2014, with fever (temperature >38°C), cough, hemoptysis, nausea, vomiting, and anorexia. She was admitted to hospital A on May 17; her condition improved, and she was discharged on May 30, 19 days after onset of symptoms.

Patient 3 was a 35-year-old female nurse assistant at hospital A who had no underlying medical conditions. Her symptoms of sore throat and productive cough were detected on May 26 as part of the investigation of the first 2 cases; co-infection with influenza A(H1N1)pdm09 was detected. Patient 3 had contact with patient 1 during her hospitalization in ICU. Patient 3 was advised to stay home and follow infection control precautions until respiratory samples tested negative.

Patient 4 was a 44-year-old male physician at hospital A with a history of chronic heart disease who had contact with patient 1 during her hospitalization in ICU. Mild respiratory symptoms developed in patient 4 on June 6; his condition deteriorated, and he was admitted to a hospital in Tehran, Iran, on June 17 with fever (temperature >38°C), sore throat, cough, dyspnea, chills, anorexia, and myalgia. Patient 4's symptoms were initially severe, but his condition improved, and he was discharged on June 21.

Patient 5 was a 67-year-old woman who was admitted to hospital A on June 6 because of exacerbation of chronic obstructive pulmonary disease. She was discharged from the hospital on June 14 and was in stable condition until severe acute respiratory infection (SARI) developed. She was readmitted to hospital A with fever (temperature >38°C), cough, and dyspnea on June 25. During her first hospitalization, the patient had close contact with another patient who had SARI but had tested negative for MERS-CoV. A respiratory sample from patient 5 was obtained on June 30, and she died on July 5.

All 5 patients were residents of Kerman Province and had no history of travel or contact with animals in the 14 days before becoming ill. Throat swab specimens and sputum samples were collected and analyzed by using real-time reverse transcription PCR (RT-PCR) performed on the basis of a previously reported method by targeting the upstream E region and open reading frame 1b of the virus (*5*). Conventional RT-PCR was conducted for the N region (*6*). The PCR products of the N region were sequenced in both directions.

Table. Patient and clinical data on 5 Middle East respiratory syndrome coronavirus infections in Kerman Province, Iran, 2014*

Patient no.	Patient age, y/sex	Date of illness onset	Hospitalization dates	Date infection confirmed	Date of death	GenBank accession no. for isolate
1	52/F	May 1	May 11–29	May 24	May 29	KM044032
2	50/F	May 11	May 17–30	May 24	NA	KM044034
3	35/F	May 26	NA	May 31	NA	NA
4	44/M	Jun 6	Jun 17–21	Jun 19	NA	KM044033
5	67/F	Jun 25	Jun 25–Jul 5	Jul 5	Jul 4	NA

*NA, not applicable.

The samples from patients 1, 2, and 4 yielded N gene sequences positive for MERS-CoV. Phylogenetic analysis showed differences between these sequences and a consensus sequence retrieved from GenBank (accession no. JX869059; Figure). All 3 sequences from these cases had polymorphisms at positions 28880 (T→C), 28941 (G→C), and 29097 (T→G). The mutation at position 28941 was nonsynonymous with an aspartic acid to histidine change. For the isolate from patient 4, another nonsynonymous mutation was observed at position 29329 (C→T), which resulted a change of tyrosine to isoleucine. In all 3 sequences, nucleotide C was detected at position 29147, as was the case with the first identified isolate of MERS-CoV. For some sequences in GenBank, this position contains T.

Conclusions

We identified a cluster of MERS-CoV infections in Iran, showing apparent person-to-person transmission but with unclear transmission routes for some patients. In this cluster, patient 1 was in close contact with a person suspected of being the index case-patient, but we were unable to verify the infection status of this patient. Patient 2 seems to have acquired the infection from patient 1. The source of infection for patients 3 and 4 was patient 1 or 2, but the source for patient 5's infection remains unknown. However, subclinical cases of MERS-CoV infection have been reported to the World Health Organization (*7*); exposure to a person with subclinical infection could explain an active infection that has an unknown route of transmission.

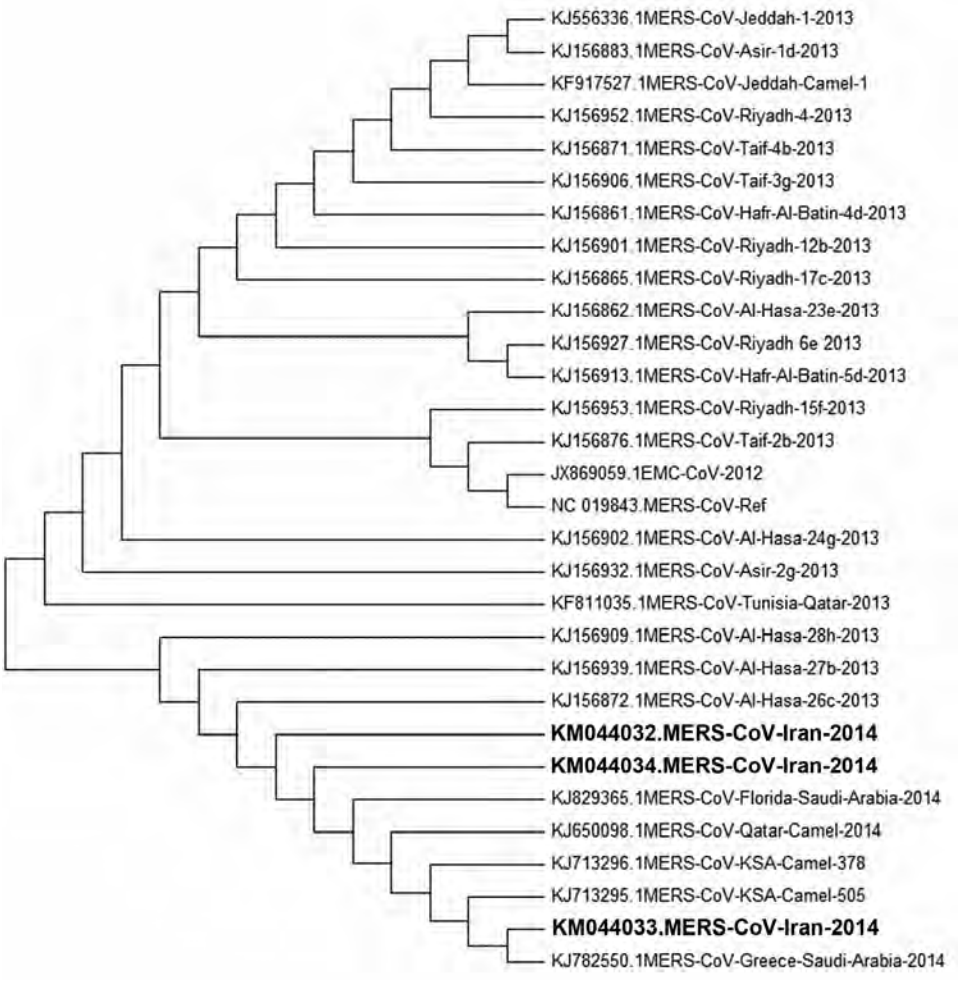

Figure. Phylogenic sequence analysis of 3 Middle East respiratory syndrome coronavirus (MERS-CoV) isolates from patients in Kerman Province, Iran (boldface), 2014, compared with sequences from GenBank (accession numbers shown). MEGA 5.2 (http://www.megasoftware.net) was used for construction of neighbor-joining tree by using the Kimura 2-parameter model with uniform rates and 1,000 bootstrap replicates.

Throat swab specimens and sputum samples were collected from all close contacts of the 5 patients in this cluster, including family members, other patients in the hospital, and health care workers. All samples were negative for MERS-CoV. Patient 1 had a pregnant daughter who was a frequent visitor during her hospitalization but who tested negative for MERS-CoV by real-time RT-PCR.

Before patient 1 was hospitalized, none of her contacts showed signs of MERS-CoV infection, but after her hospitalization (during her second week of her illness), her sister became ill and subsequently tested positive for the virus. This finding suggests that, as with severe acute respiratory syndrome, MERS-CoV is not readily transmitted during the early phases of the disease (*3*), in contrast to the other human coronaviruses, which are transmitted early in the infection (*2*). Early recognition of confirmed MERS-CoV infections and investigation of the contacts of these patients are critical for effective epidemic control. Because Saudi Arabia has reported the highest number of MERS-CoV infections, one approach for limiting the transmission of this virus may be to screen travelers from Iran who report SARI to detect MERS-CoV. However, screening of pilgrims from Iran who traveled to Mecca during the 2013 Hajj did not detect MERS-CoV infections (National Influenza Center Iran, unpub. data).

Our investigation has limitations. First, some persons who may have had MERS-CoV infection were not tested, such as the probable index case-patient with whom patient 1 had contact, the patient with SARI with whom patient 5 had contact, and the contacts of these persons. Second, we performed N gene PCRs on samples from all 5 case-patients, but results were negative for patients 3 and 5, which suggests that these samples should be tested with more specific primers.

In summary, we identified 5 cases of MERS-CoV in the same province in Iran; for several of these cases, virus transmission routes were not clearly defined. Future research should focus on clarifying routes of transmission for this virus, including the possibility of transmission from persons with subclinical infection.

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Acquisition of Human Polyomaviruses in the First 18 Months of Life

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We investigated the presence of 4 human polyomaviruses (PyVs) (WU, KI, Merkel cell, and Malawi) in respiratory specimens from a community-based birth cohort. These viruses typically were acquired when children were ≈1 year of age. We provide evidence that WU, KI, and Malawi, but not Merkel cell PyVs, might have a role in respiratory infections.

Human polyomaviruses (PyVs) JC and BK were discovered in 1971 and are believed to be acquired by a respiratory or fecal–oral route (*1*). They predominantly cause disease in immunocompromised persons (*2*). In the past 7 years, 11 new human PyVs have been described. These include WU (WUPyV), KI (KIPyV), Merkel cell (MCPyV), and Malawi (MWPyV) PyVs, all of which have been detected in respiratory secretions, particularly from children (*3*). Whether these viruses are pathogenic or simply passengers in the respiratory tract is not known. WUPyV and KIPyV were the first respiratory tract–associated PyVs and were discovered in children with acute respiratory infections (*4*,*5*).

MCPyV was identified in Merkel cell carcinoma tissue, and evidence suggested that genome integration of MCPyV initiates cell transformation (*6*). MCPyV has also been reported in respiratory samples, but potential skin or environmental contamination of respiratory samples must be considered (*7*–*9*). In 2013, MWPyV was detected in the fecal sample of a healthy child, and it has also been detected in samples from patients with gastrointestinal symptoms and in anal warts (*10*,*11*). We recently reported that MWPyV was frequently present in respiratory secretions, particularly in children <5 years of age (*12*). However, most of these studies were performed on convenience samples from acutely ill patients and included no samples or limited numbers of samples from healthy controls.

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We investigated the presence of the respiratory-associated human PyVs (WUPyV, KIPyV, MCPyV, and MWPyV) in samples collected weekly, regardless of symptoms, from healthy children in Australia during their first 18 months of life. These children were participating in a community-based longitudinal birth cohort study (Observational Research in Childhood Infectious Disease [ORChID]).

The Study

The ORChID study is an ongoing dynamic birth cohort study in Brisbane (Queensland, Australia) that has been described (*13*). The study was approved by the Human Research Ethics Committees of the Children's Health Queensland Hospital and Health Service, the Royal Brisbane and Women's Hospital, and The University of Queensland. In brief, anterior nasal swab specimens were collected at birth and weekly until the child's second birthday (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/2/14-1249-Techapp1.pdf).

The present study reports on the first 56 children to complete 18 months (censored for swab specimens collected after 530 study days) of the ORChID study. A total of 3,851 nasal swab specimens (mean 69 swab specimens/child, range 41–77 swab specimens/child) during 29,678 person-days of observation (mean 530 person-days of observation/participant, range 384–560 person-days of observation/participant). These samples were tested for WUPyV, KIPyV, MCPyV, and MWPyV by using reported real-time PCRs (*12*,*13*). Samples were screened for 13 common respiratory viruses according to the ORChID study protocol (online Technical Appendix).

A sole detection episode was defined as ≥1 consecutive swab specimens in which an individual PyV was the only virus detected, and no other viruses were reported 7 days before or after detection of the PyV. Detection of a different PyV or the same PyV after 30 days and ≥2 intervening negative samples was considered a new infection episode. During the period of the sole detection episode, clinical symptoms were broadly categorized as upper respiratory, lower respiratory, nonspecific, and gastrointestinal (online Technical Appendix). Nonspecific symptoms were only separately categorized when unaccompanied by upper or lower respiratory symptoms. Gastrointestinal

Table 1. Detection of polyomavirus by real-time PCR in a subset of 56 ORChID study participants who had weekly anterior nasal swab specimens collected for 18 months during 2010–2014*

Characteristic	WUPyV	KIPyV	MCPyV	MWPyV
No. detections (3,851 nasal swab specimens)	36	63	13	157
No. positive/no. tested (%)	14/56 (25)	26/56 (45)	13/56 (23)	31/56 (56)
Median age at primary detection, mo (range)	11 (5.2–16.6)	10 (5.6–18.6)	7 (0.07–18.8)	13 (0.9–13.1)
Median length of viral shedding, wk (range)	2 (1–4)	2 (1–5)	1 (1)	1.2 (1–3)
Co-detection with 13 other respiratory viruses (%)	18/36 (50)	31/63 (49)	3/13 (21)	52/157 (33)
Median cycle threshold (range)†	29.45 (18–37)	30.91 (18–40)	38.58 (34–40)	35.15 (30–40)

*ORChID, Observational Research in Childhood Infectious Diseases; WUPyV, WU polyomavirus; KIPyV, KI polyomavirus; MCPyV, Merkel cell polyomavirus; MWPyV, Malawi polyomavirus.
†Cycle thresholds are approximately inversely related to viral load.

symptoms were recorded in the presence or absence of respiratory symptoms.

All 4 novel PyV viruses were detected in respiratory samples from children ≤18 months of age; MWPyV was the predominant virus (157 positive detections) (Table 1). A total of 23% (13/56 for MCPyV) and 56% (31/56 for MWPyV) of children had ≥1 positive result for 1 of the PyVs. WUPyV, KIPyV, and MWPyV were initially detected when the child was ≈1 year of age, and each virus was detected for a mean of 2 consecutive weeks after primary detection. MCPyV was detected less frequently; primary detections occurred in children ≈7 months of age, which was earlier than detection of WUPyV, KIPyV, and MWPyV ($p<0.001$, by Mann-Whitney 2-tailed test with pairwise comparisons of ages). MCPyV was not seen in any consecutive swab specimen collections.

WUPyV (50%, 18/36) and KIPyV (49%, 31/63) were commonly detected with other respiratory viruses. MWPyV (33%, 52/157) and MCPyV (21%, 3/13) had lower semiquantitative viral loads ($p<0.001$, by Mann-Whitney 2-tailed test with pairwise comparisons of cycle thresholds), and were less frequently detected with other respiratory viruses (Table 1; online Technical Appendix).

Symptoms were reported during sole detection episodes for WUPyV, KIPyV, and MWPyV, but not for MCPyV (Table 2). During symptomatic episodes, numerous overlapping respiratory virus detections were commonly observed, which made sole detection episodes of PyV too rare to be considered in a formal statistical analysis. However, sole detection episodes corresponded to parental reporting of clinical symptoms for 57% (4/7) of WUPyV and 36% of KIPyV (5/14) and MWPyV (13/36) infection episodes (Table 2). Most symptoms reported during the sole detection episodes were upper respiratory (Table 2).

Conclusions

Primary acquisition of WUPyV, KIPyV, and MWPyV occurred most commonly when children were ≈12 months of age, which is consistent with previous serologic data showing PyV primary infection in children at an early age (*3*). MCPyV was also detected within this cohort, but age, frequency of detection, and lower levels of viral shedding contrasted with the findings for WUPyV, KIPyV, and MWPyV. This finding is supported by other retrospective studies that showed that MCPyV is more frequently detected in the respiratory tract of adults (*7*). Previously reported high co-detection rates of WUPyV and KIPyV with other respiratory viruses (>70%) have hampered efforts to associate these viruses with clinical symptoms (*3*,*14*). However, we observed a lower rate of PyV co-detection, which enables us to examine the association of PyV sole detection episodes with symptoms. This examination showed that most symptoms were upper respiratory, although gastrointestinal symptoms were also reported during WUPyV, KIPyV, and MWPyV sole detection episodes.

A higher viral load was observed for WUPyV and KIPyV than for MWPyV and MCPyV. Although use of cycle thresholds as a semiquantitative marker has some limitations, thresholds suggest that WUPyV and KIPyV actively replicate in the respiratory tract and may account for the higher rate of reported symptoms with sole detection of

Table 2. Characteristics of persons infected with polyomavirus in a subset of 56 ORChID study participants who had weekly anterior nasal swab specimens collected for 18 months during 2010–2014*

Characteristic	WUPyV	KIPyV	MWPyV
No. sole detection episodes	9	14	36
No. sole detection episodes with symptoms/no. tested, %	4/7 (57)	5/14 (36)	13/36 (36)
No. sole detection episodes without symptoms	3	9	23
Symptoms of LRTI	0	0	0
Symptoms of URTI	4	3	9
Symptoms of URTI and LRTI	0	0	2
Only nonspecific symptoms	0	1	2
Diarrhea/vomiting	1	1	1
Symptoms not available	2	0	0

*ORChID, Observational Research in Childhood Infectious Diseases; WUPyV, WU polyoma virus; KIPyV, KI polyomavirus; MWPyV, Malawi polyomavirus; LRTI, lower respiratory tract infection; URTI, upper respiratory tract infection.

WUPyV. A previous study in the Netherlands also reported a higher symptom association of WUPyV than KIPyV, but MWPyV was not investigated in that study (*15*). MWPyV was first detected in fecal specimens from healthy children, but we found that MWPyV was the most prevalent PyV detected in respiratory specimens and that it was associated with upper respiratory infection symptoms in >33% of sole detection episodes. We found that 1/36 participants had gastrointestinal symptoms during an episode of MWPyV sole detection, which is an observation that warrants further investigation.

MCPyV is shed from healthy skin, and MWPyV has been detected in anal warts. Thus, a limitation of this study is potential cutaneous contamination of swab specimens by the parent or child during sample collection. Although the complex nature of virus acquisition and overlapping intervals of virus detection confound the association of PyV sole detection episodes with particular symptoms, our data show that WUPyV, KIPyV, and MWPyV, but not MCPyV, are frequently detected within the respiratory tract of healthy children <18 months of age and are associated with mild upper respiratory symptoms.

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Influenza D Virus in Cattle, France, 2011–2014

Mariette F. Ducatez, Claire Pelletier, Gilles Meyer

A new influenza virus, genus D, isolated in US pigs and cattle, has also been circulating in cattle in France. It was first identified there in 2011, and an increase was detected in 2014. The virus genome in France is 94%–99% identical to its US counterpart, which suggests intercontinental spillover.

Recent studies in the United States have identified a new genus within the family *Orthomyxoviridae*, tentatively named *Influenzavirus D* (*1*). The new pathogen, C/swine/Oklahoma/1334/2011 (C/OK), was first identified in pigs with influenza-like illness and was only moderately related to previously characterized influenza C viruses (≈50% overall homology between C/OK virus sequence and its closest related sequences). Like human influenza C virus, C/OK harbored 7 genomic segments, whereas influenza A and B viruses have 8. C/OK virus genome was more distant from influenza C virus genomes than influenza A genomes are from influenza B virus genomes. In hemagglutination inhibition assays, cross-reactivity between antibodies against C/OK virus and human influenza C virus was lacking, which again suggests a new genus in the family *Orthomyxoviridae* (*2*). C/OK-like viruses were also isolated from cattle in the United States in 2013 (*1*) and China in 2014 (*2*).

C/OK was shown to replicate in ferrets, the animal model of choice for studying influenza virus in humans, suggesting that humans could be infected with C/OK-like viruses. Thus, the host range and geographic distribution of C/OK-like viruses (influenza D virus) needs to be investigated. Because cattle have been suggested as the reservoir for this novel influenza virus (*1*), we screened bovine samples in France for influenza D virus and characterized the virus from positive specimens.

The Study

Bovine lung fragments, deep nasal swab specimens, and trans-tracheal aspiration liquids were submitted to the Laboratoire Départemental d'Analyses de Saône-et-Loire (Mâcon, France) and tested for classical respiratory pathogens. The Unité Mixte de Recherche, Interactions Hôtes—Agents Pathogènes 1225 (Toulouse, France), received and tested 134 samples by using real-time reverse transcription PCR for influenza D virus as previously described (*3*). We tested 25 archived samples per year for 2010–2013 and 34 samples collected during January–March 2014. Six (4.5%) were positive for influenza D virus: 1 each in 2011 and 2012 and 4 in 2014; cycle threshold (C_t) values ranged from 15 to 35 (Table 1, http://wwwnc.cdc.gov/EID/article/21/2/14-1449-T1.htm). Coinfections were detected with *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, bovine respiratory syncytial virus, and/or bovine herpesvirus 1 in 4 of the influenza D–positive specimens. Two samples (nos. 5831 and 5920, collected in 2014) were negative for all tested respiratory pathogens, despite reports of clinical signs in the animals (Table 1).

The specimen with the lowest C_t value, D/bovine/France/2986/2012 (C_t 15) was selected for further molecular characterization, and its full genome was amplified by PCR (primers in Table 2) and sequenced on a 3130XL Applied Biosystems capillary sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were submitted to EMBL (LN559120–LN559126).

The 7 gene segments of D/bovine/France/2986/2012 clearly clustered with US influenza D strains from pigs and cattle (C/OK, C/bovine/Minnesota/628/2013, C/bovine/Minnesota/729/2013, and C/bovine/Oklahoma/660/2013) (Figure), which suggests a common origin of these new influenza viruses. We found no evidence of reassortment between influenza C and D (C/OK-like) viruses. In addition, the splicing pattern of the matrix gene segment and the reduced 5-N-acetyl binding pocket in the hemagglutinin-esterase (HE) protein of D/bovine/France/2986/2012 was similar to that of C/OK and different from that of human influenza C virus, confirming the similarity of D/bovine/France/2956/2012 and the newly described swine and bovine US influenza D virus strains. The estimated ranges of evolutionary distances (in number of substitutions per site using the maximum composite likelihood model) between D/bovine/France/2986/2012 and the 4 US influenza D viruses ranged from 0.8 to 5.7% and were as follows: 1.9%–2.1%, 0.8%–0.9%, 2.1%–2.5%, 2.3%–2.7%, 1.8%–3.8%, 3.6%–4.2%, and 5.1%–5.7% for polymerase basic (PB) 2, PB1, polymerase 3/polymerase acidic, nucleoprotein, matrix, nonstructural protein, and HE gene segments, respectively.

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Table 2. Primer sets used for PCR amplification and sequencing of the full genome of influenza D/bovine/France/2986/2012

Gene	Forward primer, 5′ → 3′	Reverse primer, 5′ → 3′
Hemagglutinin-esterase	FluD_HE-1F: AGCATAAGCAGGAGATTTTCAAAG	FluD_HE-745R: GCACTACATGCTTGTTGC
	FluD_HE-667F: GTTTGTGGGACTGAGCAATC	FluD_HE-1350R: CCCTGCTTGCGGTATTATC
	FluD_HE-1267F: CCCAAGTATGGCAGATG	fluD_HE-2042R: GCAAGGAGATTTTTTCTAAGATT
Matrix	FluD-MP-8F: GCAGAGGATATTTTTGACGC	FluD-MP-670R: CCCATATGCTATTCTTGCCAG
	FluD-MP-602F: AAAAAAGAGGCCCAGGCAC	FluD-MP-1212R: GCAAGAGGATTTTTTCGCG
Nucleoprotein	FluD-NP-1F: GGCATAAGCAGGAGATTATTAAGC	FluD-NP-949R: TAAAGGCTCTTACTCCAGAATA
	FluD-NP-849F: GCCTTGGTCAATGTGGCTG	FluD-NP-1717R: GGGGACTGCAACAGAACCA
Nonstructural protein	FluD-NS-8F: GCAGGGGTGTACAATTTCAAT	FluD-NS-804R: TCGAAACTGACTTGATTTCATCC
Polymerase 3	FluD-P3–1F: GGCATAAGCAGGAGATTTA	FluD-P3–759R: TTTTCTTCTAGATGTTCCAGTTTGA
	FluD-P3–677F: AAAAGAAATCAGGCTGAATGC	FluD-P3–1467R: CCAAACAAACAGTCAGTTGA
	FluD-P3–1394F: CCCGGAAAGGTCAAGATAG	FluD-P3–2184R: GGAGATTTTTAACATTACAAGGCC
Polymerase basic 1	FluD-PB1–1F: GGCATAAGCAGAGGATTTTAT	FluD-PB1–736R: TTTTCCTCTTTCTCCGTC
	FluD-PB1–631F: AAAAATGAAGTCTCCAACATTG	C/OK-Rev (*2*)
	C/OK-Fwd (*2*)	FluD-PB1–2317R: GATTTTTCTGTTATTAAACAACGC
Polymerase basic 2	FluD-PB2–1F: GGCATAAGCAGAGGATGTC	FluD-PB2–882R: CCCTTATCTTCTCTGCTGG
	FluD-PB2–796F: AAAAGAAGAGAGATGTTAGAGC	FluD-PB2–1776R: TTTTACCCATTATCAAAGCAGG
	FluD-PB2–1724F: GAAAGAATAAACACTGATGATG	FluD-PB2–2353R: GAGGATTTTTTCAATGTGCTTC

We also identified unique features in D/bovine/France/2956/2012 genome. Forty unique amino acid substitutions were identified throughout the genome, but the limited available data on influenza D genomes make a functional interpretation of the substitutions difficult to determine. In addition, although the HE proteins of human influenza C and C/OK viruses contain 7 and 6 potential glycosylation sites, respectively, D/bovine/France/2986/2012 has just 5: at positions 28, 54, 146, 346, and 613 (ATG numbering), identical to 5 of the 6 identified for C/OK virus. For influenza A viruses, modifications of N-linked glycosylation sites in the globular head of the hemagglutinin protein have been linked to changes in virulence, antigenicity, receptor-binding preference, fusion activity, and immune evasion (*4*). For example, an increase in glycosylation site numbers has been associated with early stages of influenza A(H1N1) virus evolution, and changes in the positional conversion of the glycosylation sites have been associated with later evolutionary stages of the virus (*5*). The missing potential glycosylation site in D/bovine/France/2986/2012 is located at position 513, probably likely in the F3 = HE2 fusion domain of the protein (*6*) and not in the globular head of the protein. Thus speculating on the putative time course of virus emergence between C/OK-like and D/bovine/France/2986/2012-like strains is difficult. Further studies are needed to understand the phenotype(s) associated with aa substitutions in influenza D viruses.

Conclusions

Webster et al. suggested the existence of a common ancestor for influenza A, B, and C viruses and a more recent common ancestor to influenza A and B viruses only considering the different genome organizations between influenza A/B and influenza C viruses (*6*). Sheng et al. recently estimated the time of divergence between influenza C and D virus gene segments at 334 years for PB1 to 1,299 years for HE (*7*). The time of emergence and evolutionary rate of influenza D viruses need to be examined as more data become available. A puzzling question raised by our current study is the geographic origin of influenza D strains: were cattle in France contaminated by their North American counterparts or vice versa? Did co-evolution occur? Did the pathogen originate from a distinct location or from a distinct host?

Retrospective studies with archived samples would help date the emergence of influenza D viruses and enable an understanding of their evolution. In addition, the geographic prevalence still needs to be investigated.

The pathogen may have spread to swine and cattle in recent years only; efforts should be made to find the virus host range and its reservoir species and to evaluate the public health relevance of this new pathogen. Finally, surveillance projects with larger cohorts, as well as experimental infections, need to be conducted before 1) the causality between respiratory symptoms and influenza D virus infection in cattle can be established, 2) recommendations on samples to collect can be given, and 3) prevalence can be compared in different geographic areas. Although the causative agent(s) of some respiratory infections in the field remain(s) unknown (G. Meyer, pers. comm.) and although 2 of the positive specimens in our study originated from young cattle with respiratory symptoms but no identified respiratory pathogen, further studies are also warranted to provide an understanding of the pathobiology of influenza D virus in cattle and its putative role in complex bovine respiratory disease.

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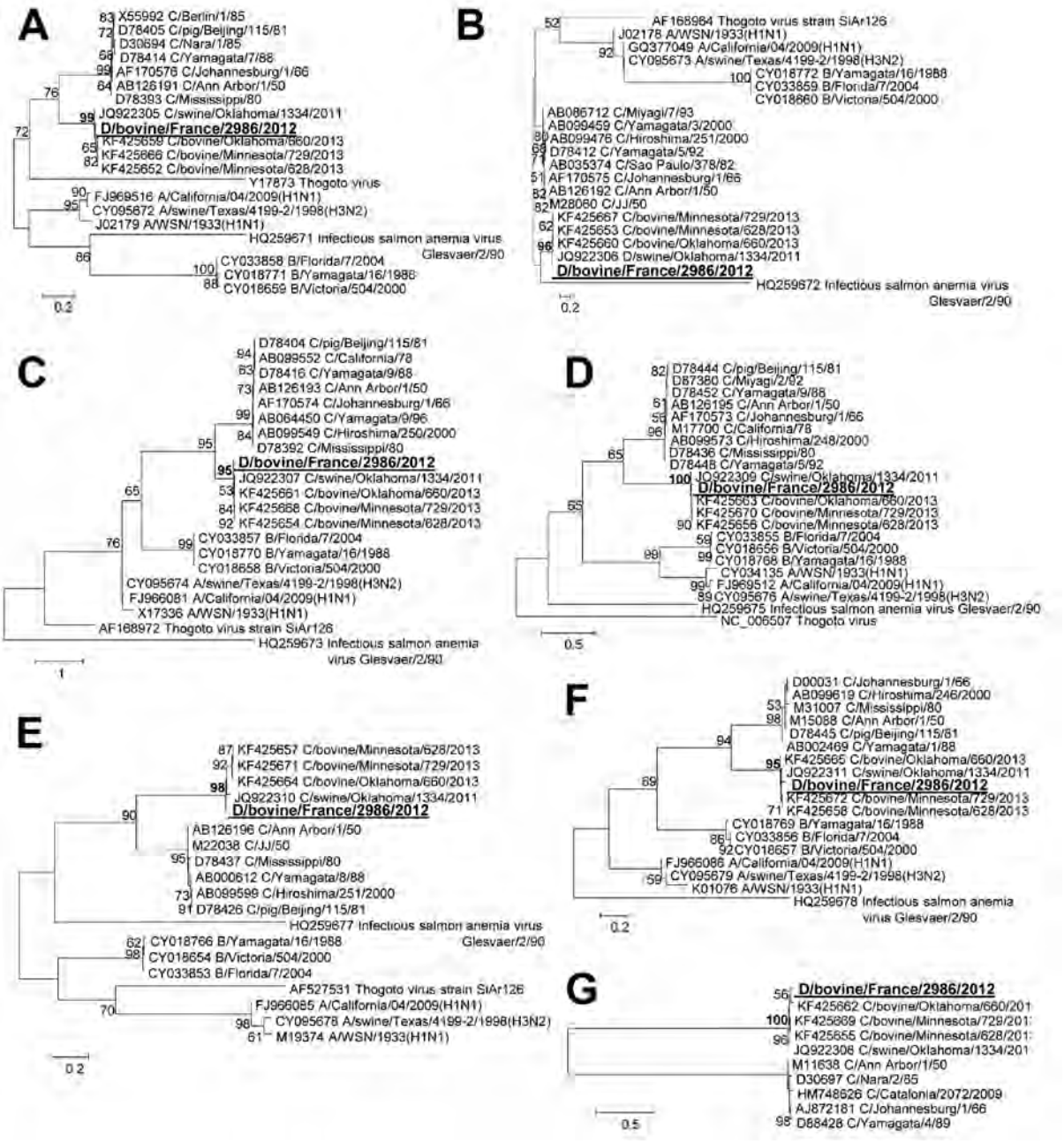

Figure. Phylogenetic trees of the 7 gene segments of D/bovine/France/2986/2012 influenza virus at the nucleotide level. A) PB2. B) PB1. C) P3/PA. D) Nucleoprotein E) P42/Matrix. F) Nonstructural protein. G) Hemagglutinin-esterase. Maximum-likelihood analysis with 500 bootstrap replicates (bootstrap values >75 are indicated on the tree nodes). The gene sequences of D/bovine/France/2986/2012 (in large bold underlined font) were compared with representatives of all the *Orthomyxoviridae* genera: all the viral strains used in (*1*). P, polymerase, nucleoprotein, PB, polymerase basic Scale bars indicate nucleotide substitutions per site.

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Outbreak-Associated Novel Avipoxvirus in Domestic Mallard Ducks, China

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To the Editor: In December 2013, an unidentified disease in domestic mallard ducks (*Anas platyrhynchos*) occurred in Guangxi Province, China. Rates of illness in adult male and female ducks were 50%–70% and 5%–30%, respectively, in different flocks. No deaths were observed. Clinical signs included cutaneous nodules on the birds' eyelids, beaks, and legs. All classical endemic viruses and bacteria, including avian influenza virus, avian paramyxovirus 1, duck enteritis virus, *Riemerella anatipestifer*, and *Escherichia coli*, were excluded as causative agents by PCR or bacteria isolation. During investigation of the illness, we isolated a novel duck-pathogenic avipoxvirus (APV) from skin nodules of the affected ducks.

APVs, which contain a double-stranded DNA genome, are members of the genus *Avipoxvirus*, family *Poxviridae* (*1*). They naturally infect 232 species in 23 orders of wild and domestic birds worldwide (*2*). APV infections have 2 forms, cutaneous and diphtheritic, and can occur in either form or both forms. Cutaneous APV infection is characterized by nodular lesions on featherless areas of the body; diphtheritic APV infection usually results in higher death rates and produces nodular lesions on the mucous membranes of the mouth, esophagus, and/or trachea (*1*). In China, APV infections have been found in chickens, pigeons, turkeys, quail, and geese (*3*) but not in domestic ducks. APV antibodies were found once in a wild mallard duck (*4*), but no further etiologic and histopathologic evidence was found.

During December 2013–January 2014, farmers in 3 counties (Daxin, Xilin, and Tiandong; distance between each county 70–230 km) of Guangxi Province almost simultaneously reported to local veterinary services an unidentified disease in domestic mallard ducks. A total of 19 farms where domestic mallard ducks were raised (15 in Tiandong, 3 in Daxin, and 1 in Xilin) and 50,000 birds were affected. Affected ducks behaved and ate normally. Nodular lesions with scabs appeared on the unilateral or bilateral eyelids of affected ducks and on featherless regions on beaks and/or legs of 10% of affected ducks in some flocks (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/21/2/14-0215-Techapp1.pdf). No lesions were found in the digestive and respiratory tracts of affected ducks. Histopathologic examination of skin nodules using hematoxylin and eosin stain showed proliferative and necrotic dermatitis, with ballooning degeneration of keratinocytes, and large, eosinophilic ring-shaped Bollinger bodies (online Technical Appendix Figure 2). Brick-shaped 330 × 280 × 200–nm virus particles with irregular pipe-shaped surface structures (online Technical Appendix Figure 3), consistent with those of members of genus *Avipoxvirus*, were observed by electron microscopy. Ultrastructurall examination revealed that cytoplasm within degenerating epithelium contained inclusions comprising viral particles. The particles had a dumbbell-shaped central core, lateral bodies, and a convoluted outer membrane (online Technical Appendix Figures 4, 5). Virus isolation from skin nodules was conducted on duck chorioallantoic membranes of specific pathogen–free embryonated 11-day-old duck eggs by using the method described by Joklik (*5*). At 7 days after infection, small pocks in chorioallantoic membranes and membrane thickening were observed. Three isolates from Xilin, Daxin, and Tiandong were designated as APV-XL, APV-DX, and APV-TD, respectively.

We amplified the partial sequences of *P4b* gene (*fpv167* locus) and DNA polymerase gene (*fpv094* locus) of the aforementioned 3 isolates using the specific primer pairs (P1: 5′-CAGCAGGTGCTAAACAACAA-3′, and P2: 5′-CGGTAGCTTAACGCCGAATA-3′ for P4b; PPolF: 5′-GGCYAGTACKCTTATYAAAGG-3′, and PPolR: 5′-CGTCTCTACGTGTTTCGCT-3′ for polymerase gene) (*6*,*7*). The sequences obtained (GenBank accession nos. KJ192189–KJ192191 for P4b gene, KM281932–KM281934 for polymerase gene) were aligned with published APV sequences. Among them, sequences from 3 Guangxi isolates were 100% nt identical to each other, suggesting they are the same virus. We further determined phylogenetic relationship using the Bayesian approach based on a general time reversible model with a γ distribution (GTR+G) implemented in MrBayes 3.2, as described by Gyuranecz et al. (*7*) and Ronquist et al. (*8*). The phylogenetic tree based on the concatenated sequences (981 bp, n = 62) of partial P4b (426 bp) and polymerase gene (555 bp) showed that Guangxi isolates group into a new cluster within subclade A5, which comprises viruses isolated from wild waterfowl, including mallard ducks, trumpeter swans, mottled ducks, redhead ducks, and blue-winged teal from North America (*6*,*7*,*9*). Estimated with MEGA according to the methods of Gyuranecz et al. (*7*) and Tamura et al. (*10*), we found that mean genetic distances of P4b

genes, polymerase genes, and the concatenated sequences, between North American and Guangxi isolates were 0.002 ± 0.002 SE, 0.034 ± 0.004 SE, and 0.019 ± 0.003 SE, respectively (Figure).

Our findings highlight the possibility that APV has been recently introduced by wild waterfowl in the Northern Hemisphere into domestic mallard ducks. Further study is needed to determine the pathogenicity of this virus on other commercial poultry species and its influence on the poultry industry and wildlife protection.

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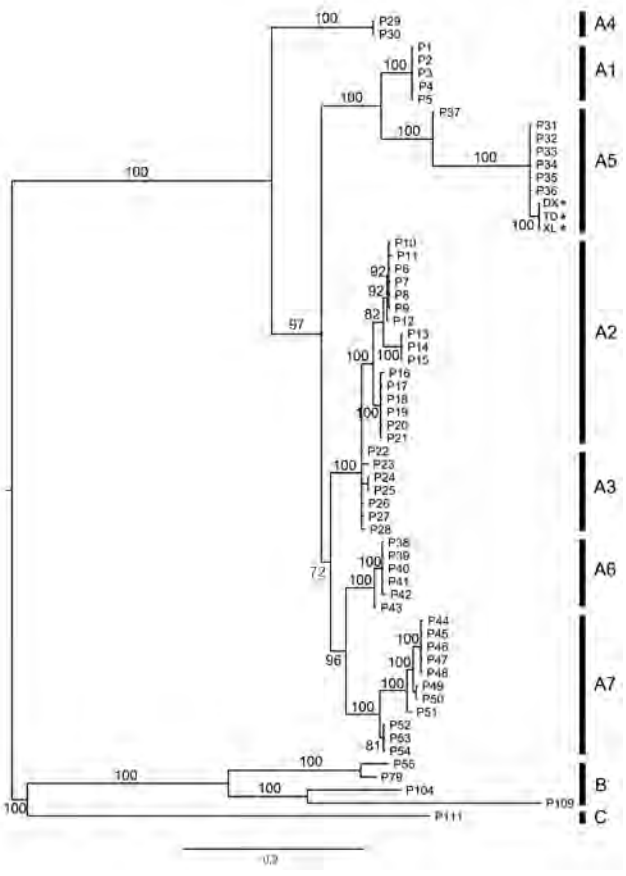

Figure. Bayesian phylogeny of concatenated DNA sequences (981 bp, n = 62) from genes encoding 4b core and DNA polymerase proteins of avipoxviruses. Posterior probability values of the Bayesian trees (1,000 replicates) are indicated. Asterisks (*) indicate sequences obtained in this study. Avipoxvirus clades A–C and subclades are labeled according to the nomenclature of Jarmin et al. (*6*) and Gyuranecz et al. (*7*). Reference sequences used in this analysis are chosen from the study of Gyuranecz et al. (*7*). Scale bar indicates nucleotide substitutions per site.

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Orientia tsutsugamushi in Lung of Patient with Acute Respiratory Distress Syndrome, France, 2013

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To the Editor: Pulmonary involvement is a well-documented complication of scrub typhus caused by *Orientia tsutsugamushi* (*1*). Lung involvement manifests as bronchitis and interstitial pneumonitis of various grades that

progress to acute respiratory distress syndrome (ARDS), a serious complication that occurs in ≈11% of scrub typhus patients (*2*). The death rate among scrub typhus patients with ARDS can reach 25% (*3*). Older age, thrombocytopenia, and the presence of early pneumonitis have been proposed as risk factors for the development of ARDS in scrub typhus patients (*3*). We report the detection and culture of *O. tsutsugamushi* in a bronchoalveolar lavage specimen from a patient with scrub typhus–associated ARDS.

A 50-year-old woman from Lyon, France, was admitted to the hospital in November 2013 with fever (39°C), dizziness, diarrhea, dyspnea, and nonproductive cough. The woman, who had just returned from travel to a jungle in Laos, reported that the fever and diarrhea had begun immediately before her return home. Examination revealed that she had an oval eschar on her back and a faint maculopapular rash. Laboratory values showed elevated C-reactive protein and liver enzyme levels, lymphocytopenia, and thrombocytopenia. Extensive microbiological testing was done, including tests to rule out malaria, dengue, viral hepatitis, and leptospirosis; all results were negative. *Salmonella* sp. infection was suspected, and treatment with ofloxacin was started.

On hospitalization day 5, the patient showed development of septic shock, renal failure, and ARDS. She was transferred to an intensive care unit, and treatment with ceftriaxone was started. On hospitalization day 6, a skin biopsy of the eschar (2 mm × 5 mm) and blood, serum, cerebrospinal fluid (0.5 mL), and bronchoalveolar lavage (1 mL) samples were obtained and sent to the National Reference Center for Rickettsiae (Marseille, France) for analysis. Total genomic DNA was extracted (Biorobot EZ1 Workstation; QIAGEN, Courtaboeuf, France) from 200 mL of each sample and used as template in a real-time PCR, which used primers and probes targeting a 47-kDa outer membrane protein gene, as described (*4*). Blood, skin biopsy, and bronchoalveolar lavage samples were positive for *O. tsutsugamushi*; the cerebrospinal fluid sample was negative. The serum sample was positive for *O. tsutsugamushi* by indirect immunofluorescence assay (serotypes Gilliam, Kuroki, Sennetsu, and Kawasaki) (*5*) and positive for *O. tsutsugamushi* IgM. Oral doxycycline (200 mg/day) was started on hospital day 7; the fever resolved 4 days later.

For culture, the positive samples were directly inoculated into monolayers of L929 cells, as described (*6*). Cultures of blood and skin biopsy samples were negative, but *O. tsutsugamushi* was isolated from the bronchoalveolar lavage sample after 40 days of culture (Figure); 500 µL of bronchoalveolar lavage fluid was used for culture. We performed PCR amplification and sequencing of the isolate, targeting a 372-bp fragment of the 56-kDa protein gene, and compared the sequences with *O. tsutsugamushi* 56-kDa protein–encoding gene sequences available in GenBank (*7*). The sequences showed 99% similarity with strains Jin/2012 and Zhou/2013 (GenBank accession nos. KJ001159 and KJ001163, respectively), which were obtained from febrile patients in Zhejiang Province, China, and have not been linked to a reference serotype (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/21/3/14-0860-Techapp1.pdf). In light of the test results and the patient's recent travel to Laos, she was given a diagnosis of *O. tsutsugamushi* infection–associated ARDS.

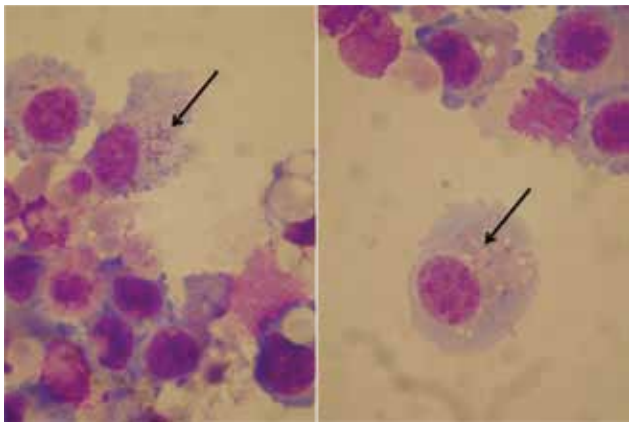

Figure. *Orientia tsutsugamushi* (arrows) in culture of bronchoalveolar lavage fluid from a patient with acute respiratory distress syndrome (Diff-Quick stain, VWR International, France). Original magnification ×100. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/21/2/14-0860-F1.htm).

Our isolation of *O. tsutsugamushi* in bronchoalveolar lavage fluid from a patient with scrub typhus shows that this bacterium can be present in such samples. We also showed that skin biopsy and bronchoalveolar lavage samples can be used for the diagnosis of scrub typhus. To be suitable for culture, samples must be collected as early as possible in the disease course. In this case, blood and skin biopsy samples were obtained late in the disease, which may explain why *O. tsutsugamushi* was not isolated from these samples. Endothelial cells are the target cells of *O. tsutsugamushi* in the lung (*8*), and it has been proposed that ARDS in scrub typhus is associated with a cytokine increase as part of the immune response to *O. tsutsugamushi* infection (*9*).

Rickettsial diseases are increasingly being diagnosed in international travelers: one report showed that 2% of imported fevers are caused by rickettsioses, and hospitalization was necessary for the 38% of *O. tsutsugamushi*–infected travelers (*10*). The diagnosis of rickettsial infections is challenging because many physicians are unfamiliar with these diseases. However, the diagnosis of scrub typhus in patients with ARDS is critical for initiating appropriate and timely doxycycline treatment. In the case reported here, a diagnosis of scrub typhus was not suspected even though the patient had compatible exposure and travel histories

and clinical findings consistent with the disease. The delay in diagnosis led to a life-threatening condition for the patient. Physicians in areas where scrub typhus is nonendemic should have a high index of suspicion for rickettsial infections in patients with recent travel histories to areas where the disease is endemic and consider treatment with tetracyclines whenever rickettsial infection is suspected. Furthermore, the potential for aerosol transmission of *O. tsutsugamushi* from patients with scrub typhus–associated ARDS to health care workers should be evaluated.

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Clustered Cases of *Oestrus ovis* Ophthalmomyiasis after 3-Week Festival, Marseille, France, 2013

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To the Editor: Ophthalmomyiasis is a zoonosis generally caused by *Oestrus ovis*, a fly that lays eggs on the eye of its host. The hatched larvae cause irritation, and left untreated, the infestation can lead to blindness (*1*). The disease is rare and is mainly reported as sporadic cases in pastoral areas where the population is in close contact with common reproductive hosts of the fly, such as sheep and goats in the Middle East (*2*), Southeast Asia (*3*,*4*), and the Mediterranean Basin (*5*,*6*). Only limited ophthalmomyiasis outbreaks have been reported around the Mediterranean Sea (*7*). A century ago, the Provence region of southern France was a pastoral area, where twice a year, large herds of sheep migrated between the pastures in the mountains north of the region and the grassland plains in the southwest. These migrations were termed *transhumance*. In 2013, Marseille metropolis, the largest urban area in Provence, was chosen as the yearly "European Capital of Culture." In this context, from May 17 to June 9, a large-scale *transhumance* event took place, which featured the gathering of huge flocks of sheep (Figure) that had passed through many towns in the vicinity of Marseille. *La transhumance* culminated in a parade through downtown Marseille, where 600 horseback riders converged with flocks of 3,000 sheep and goats, and >300,000 spectators gathered.

From the last week of June through the third week of July, 4 cases of ophthalmomyiasis were reported in the area surrounding Marseille (Figure). Only 1 case had occurred in the region during the previous 5 years. The first case-patient was a 45-year-old female teacher, who lived and worked in Allauch, located in the immediate suburbs, 21 km (≈13 mi) east of Marseille. On June 25, while on the school playground, she described feeling a fly hit her right eye. In the evening, itching and irritation of the eye prompted her to seek referral to an ophthalmologic emergency center. Examination concluded the presence of small mobile larvae inside the eye, which were identified as *O. ovis* (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/21/2/14-0974-Techapp1.pdf).

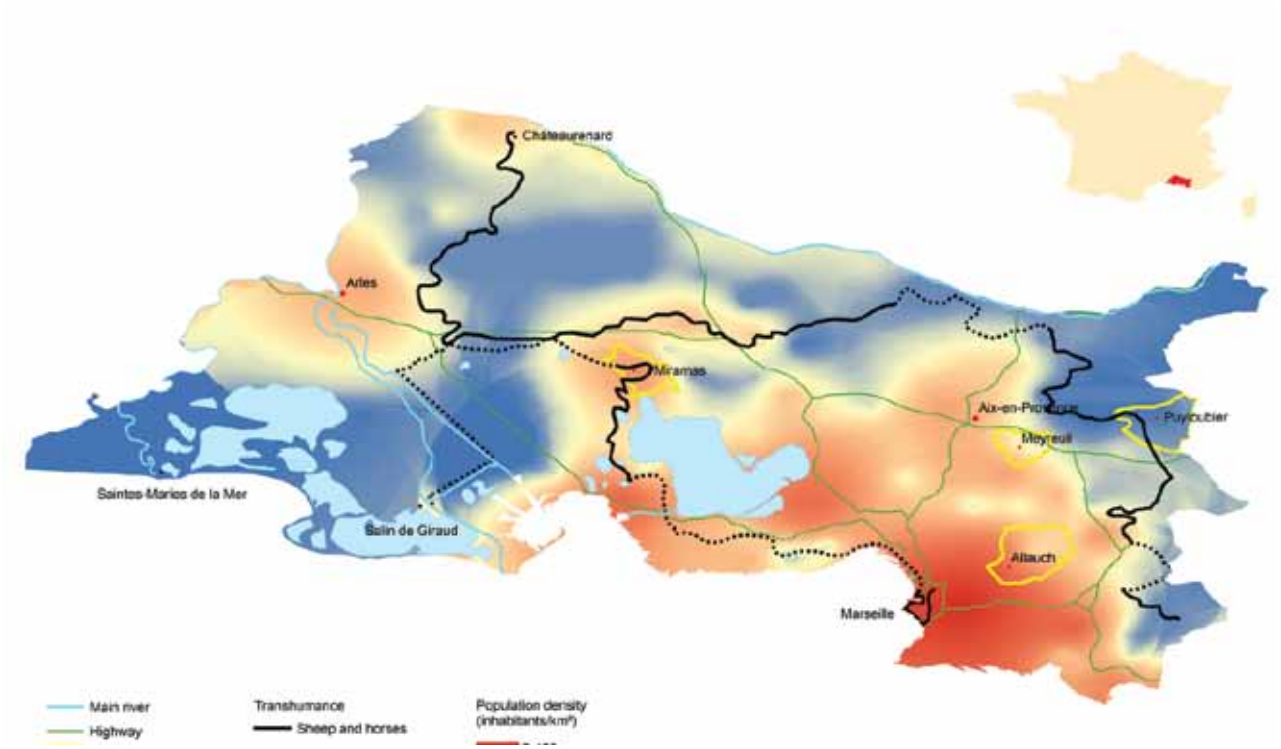

Figure. Map of *la Transhumance* routes and locations of *Oestrus ovis* ophthalmomyiasis case-patients in areas surrounding Marseilles, France, 2013. Inset shows location of the area in France. A color version of this figure is available online (http://wwwnc.cdc.gov/EID/article/21/2/14-0974-F1.htm).

The second case-patient was a retired female farmer, 67 years of age, living near Puyloubier, 49 km (≈30 miles) north of Marseille, who reported being stung on the eye by an insect during the morning of July 6. In the afternoon, the eye became painfully inflamed. On the next day, an ophthalmologist performed an excision and extracted *O. ovis* larvae from the eye.

The third case-patient was a 43-year-old female nurse's aide residing in Meyreuil, which is ≈30 km (≈18 mi) north of Marseille. On July 13, while she was on her terrace, she described ocular trauma by a fly. The next day, she sensed a foreign body in her eye, and she consulted an ophthalmologist. A simple excision led to the identification of an *O. ovis* larva.

The final case-patient was a 28-year-old male mason. On July 22, while working in Miramas, approximately 63 km (≈39 miles) he experienced trauma to his left eye. As the pain persisted, he consulted an ophthalmology facility that same evening. On examination, the presence of an *O. ovis* larva was confirmed, and an ablation was performed. All patients recovered without consequences.

O. ovis, also called sheep nasal botfly, is a fly of the class Insecta, order Diptera and family *Oestridae*. It is a cosmopolitan parasite that infects the nasal sinuses of sheep and goats. During the summer and early autumn, the adult female flies are active, laying and retaining eggs until they hatch. The fly then ejects many first-instar larvae onto the nostril of the host. The *O. ovis* larvae grow in the mucus of the nasal sinus until mature; they are then released from the nostrils when the infected host sneezes (*8*). The larvae pupate in the soil for 4–8 weeks, form a chrysalis, in which they morph into adults, and then emerge. Occasionally, *O. ovis* can infest humans, which become an intermediate accidental host (*7*). The 4 cases of ophthalmomyiasis described in this report occurred in a restricted area during a 4-week period, which corresponds exactly to the time and location of *la Transhumance*, taking into account the 4- to 8-week time lag required for the maturation of larvae into adults. Three of these cases were directly referred to our laboratory, the regional referent parasitology laboratory. The fourth case was reported by an ophthalmology emergency unit. Note that ophthalmomyiasis is rare in Marseille; during the 5 years before *la Transhumance* of 2013, only a single case had been diagnosed in the area.

Overall, this report reminds us that bringing a large group of livestock into contact with a dense urban population may enhance the risk for transmission of zoonoses. The transmitted zoonosis in this case was oestrosis, a benign condition that can sometimes progress to blindness if untreated. However, other much more severe air-transmitted zoonotic diseases associated with sheep and goats, such as Q fever, could have been transmitted (*9*). Without questioning

the organization of such an event, which the community considers to be important from a cultural and economic point of view, public health authorities should consider and anticipate as much as possible the potential sanitary consequences of such a gathering and prepare medical staff for the potential occurrence of unfamiliar diseases.

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Meningococcal Disease in US Military Personnel before and after Adoption of Conjugate Vaccine

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To the Editor: Meningococcal disease in US military personnel is controlled by vaccines, the first of which was developed by the US Army (*1*–*5*). In 1985, the quadrivalent polysaccharide vaccine (MPSV-4) was implemented as the military standard. It was replaced during 2006–2008 by the quadrivalent conjugate vaccine (MCV-4). Every person entering US military service is required to receive this vaccine.

Meningococcal disease incidence in active-duty US military personnel, historically far above that in the general population (*6*), has decreased >90% since the early 1970s, when the first vaccine was introduced (*7*). Over the last 5 years, incidences in the military and US general populations have become equivalent (*8*). Here we update previously published data (*8*) from the Naval Health Research Center's Laboratory-based Meningococcal Disease Surveillance Program of US military personnel. Data-gathering methods and laboratory analyses of samples from personnel suspected of having meningococcal disease have been previously described (*8*). Incidences were compared by using the New York State Department of Public Health Assessment Indicator based on the methods of Breslow and Day (*9*).

During 2006–2013 in US military personnel, only 1 of the 28 meningococcal disease cases for which serogroup data are available was not serogroups C or B (8 cases each) or Y (11 cases). During that period, incidence in US military personnel of 0.271 cases per 100,000 person-years did not differ significantly ($p>0.05$) from that of 0.238 in the 2006–2012 age-matched US general population (persons 17–64 years of age) (Centers for Disease Control and Prevention [CDC], unpub. data). During 2010–2013, meningococcal disease incidence in military personnel was 0.174 cases per 100,000 person-years, compared with 0.194 in the age-matched 2010–2012 US population. Among military personnel, only 1 case each occurred in 2011 (serogroup Y) and 2012 (serogroup B), and 3 occurred in 2013 (1 each of serogroups B, C, and Y).

To measure the relative success of the 2 vaccines, we compared incidence among military personnel who

had received MPSV-4 with that of personnel who had received MCV-4. In 2006, MCV-4 was introduced to new recruits. The proportion of military personnel who had received MCV-4, rather than MPSV-4, increased from 6% of the military population (63,000 persons) in 2006 to 64% (930,000) in 2013. By 2013, a total of 99% of new vaccinations were of MCV-4. Overall incidence in personnel receiving MCV-4 was 0.298 cases per 100,000 person-years during 2006–2013, which was lower, although not significantly lower ($p>0.05$), than 0.410 cases per 100,000 person-years in MPSV-4 recipients during 2000–2013.

However, because neither vaccine covers serogroup B, excluding serogroup B cases in the vaccine-related incidence calculations might be more appropriate. Incidence in MCV-4–vaccinated personnel during 2006–2013, excluding serogroup B cases, was 0.183. Specific serogroup data are not available for 2000–2005, so to calculate non–serogroup B incidence during this period, we estimated the proportion of serogroup B cases by examining a range of estimates of serogroup B proportions derived from the true proportions in all 6-year periods during 1995–2012 in the US general population (range 21%–35%; 35% during 2000–2005) (CDC, unpub. data) and during 2006–2013 in US military personnel (range 22%–28%). Adopting (from our estimated range of serogroup B proportions) 21% as the percentage that would have made the MPSV-4–related incidence the highest, MPSV-4–related incidence (i.e., excluding serogroup B cases) during 2000–2013 would have been 0.307, which did not differ significantly from incidence of MCV-4 non-serogroup B cases ($p>0.05$). (Using higher percentages would have pushed the MPSV-4 estimate even closer to the MCV-4 incidence.) The Figure shows pooled incidence for 2000–2013.

Results of these comparisons are subject to several limitations. First, because the relative proportions of the 2 vaccines changed, a differential effect of herd immunity caused by one or the other could have differentially suppressed rates. Second, along with the decrease in the MPSV-4 population, the average time from vaccination increased relative to the period in which MPSV-4 was given, concomitant with decreasing immunogenicity. Any elevated incidence in the MPSV-4–vaccinated population since 2006 could be associated with time since vaccination. Third, the same factors involved in the decline in incidence in the US general population that began in ≈2001 might be at play in the military, confounding the vaccine effects. Fourth, as the rate of vaccine coverage in the US population increased, a higher proportion of recruits might have entered the military already vaccinated; thus, their military vaccination was essentially a booster.

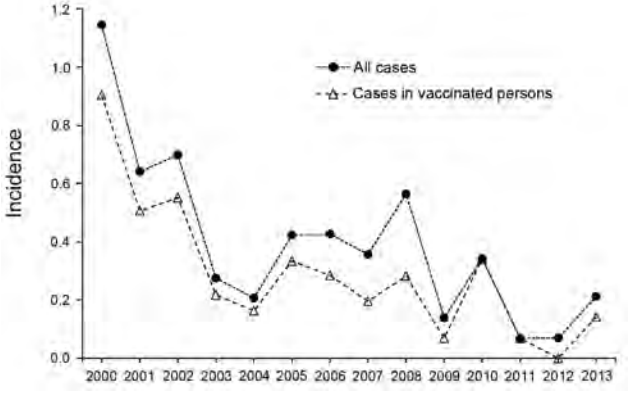

Figure. Meningococcal disease incidence per 100,000 person-years in US military personnel, 2000–2013. Incidence in vaccinated personnel shown assumes that 21% of cases during 2000–2005 were caused by *Neisseria meningitidis* serogroup B.

Meningococcal disease incidence decreased during 2000–2013. Our data suggest that cases in MCV-4–vaccinated personnel are similar to those in MPSV-4–vaccinated personnel, regardless of whether the incidence calculation includes cases caused by serogroup B (non–vaccine covered). More extensive study is needed to confirm the relative effects of the vaccines (*10*). Serogroup B accounted for 5 of the 8 cases during 2012–September 2014), and prevention of disease caused by this serotype remains a challenge.

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The meningococcal disease surveillance in the US military produces a quarterly report, which is available online: http://www.med.navy.mil/sites/nhrc/geis/Documents/MGCreport.pdf

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Chikungunya Virus Mutation, Indonesia, 2011

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To the Editor: Chikungunya virus (CHIKV) is a single-stranded, positive-sense RNA virus of ≈11.8 kb molecules (*1*) belonging to the family *Togaviridae* and genus *Alphavirus*. Genotypes of CHIKV include Asian, East/Central/South African (ECSA), and West African. CHIKV is endemic to Africa, southern Asia, and Southeast Asia and frequently causes debilitating but nonfatal illness.

CHIKV attracted global attention when a large epidemic on Réunion Island in 2005–2006 spread rapidly to other parts of the world (*1*). The predominant strain during this epidemic was the ECSA genotype with the A226V mutation of the E1 protein (*2*), the transmission of which is reported to be facilitated by *Aedes albopictus* mosquitoes (*3*). The ECSA genotype has been reported to circulate in Southeast Asia, including Malaysia, but not in Indonesia (*4*). Concern about circulating ESCA strains triggered alerts in 2009, when the Indonesian Ministry of Health reported an increasing number of chikungunya cases (3,529 cases in 2008, 83,756 in 2009) (*5*). However, only Asian genotypes were detected (*4*). We investigated recent outbreaks of CHIKV in Indonesia and genotypes of associated CHIKV strains.

After chikungunya outbreaks were reported from 6 districts in Indonesia (Tangerang, Karang Anyar, Ngawi, Jembrana, Mataram, and Kubu Raya), a team from the National Institute of Health and Research Development, Indonesian Ministry of Health, conducted field investigations from April through October 2011. This study received institutional review board approval (KE.01.06/EC/373/2011).

Serum specimens from persons with fever ≥38°C who provided signed informed consent were tested at the Virology Laboratory, Center for Biomedical and Basic Technology of Health, National Institute of Health Research and Development, in Jakarta. Molecular examination by reverse transcription PCR (RT-PCR) of acute-phase serum specimens, selective for the E1 gene, was performed as previously described (*6*). Amplicons (330 bp) were sequenced for confirmation. The entire E1 gene of 2 identified ECSA genotypes was sequenced (*7*). A cladogram was created by using MEGA version.6.06 and the neighbor-joining method (*8*). The strength of the cladogram was estimated by bootstrap analyses that used 1,000 random samplings. To determine the circulating genotype of CHIKV in Indonesia, we compared these results with other reference sequences in GenBank.

RT-PCR confirmed CHIKV in 28 (26%) of 109 samples from 5 districts: 12 (50%) in Mataram, 8 (47%) in Jembrana, 2 (40%) in Tangerang, 4 (21%) in Ngawi, and 2 (9%) in Kubu Raya. No samples from Karang Anyer were positive for CHIKV. Sequencing analysis revealed the A226V mutant (alanine to valine) ECSA genotype in 2 (7%) specimens (GenBank accession nos. KJ729851, KH729852) and the Asian genotypes (KJ729829–50, KJ729853–56) in 26 (93%) specimens. The Asian genotypes were closely related to those of CHIKV isolated from East Kalimantan, Bandung, Malaysia, and India (Figure).

The 2 cases associated with the A226V mutant ECSA genotype occurred in October 2011 in the Kubu Raya district, West Kalimantan, near the Malaysia border. Because both patients had no history of travel to Malaysia, where outbreaks involving the ECSA genotype had been reported, this finding demonstrates the emergence of the CHIKV A226V ECSA genotype in Indonesia. The 2008 nationwide outbreak of chikungunya in Malaysia proved that A226V mutation enhances transmissibility of CHIKV by *Ae. albopictus* mosquitoes (*9*). Population movement from this region might contribute to the spread of this virus to Indonesia, which is a concern because of the higher transmissibility of the mutated ECSA strain through the *Ae. albopictus* mosquito vector, which is prevalent throughout Indonesia.

That ECSA genotypes were not found in other districts during this investigation would suggest that this strain was not the source of the 2008–2009 outbreaks in

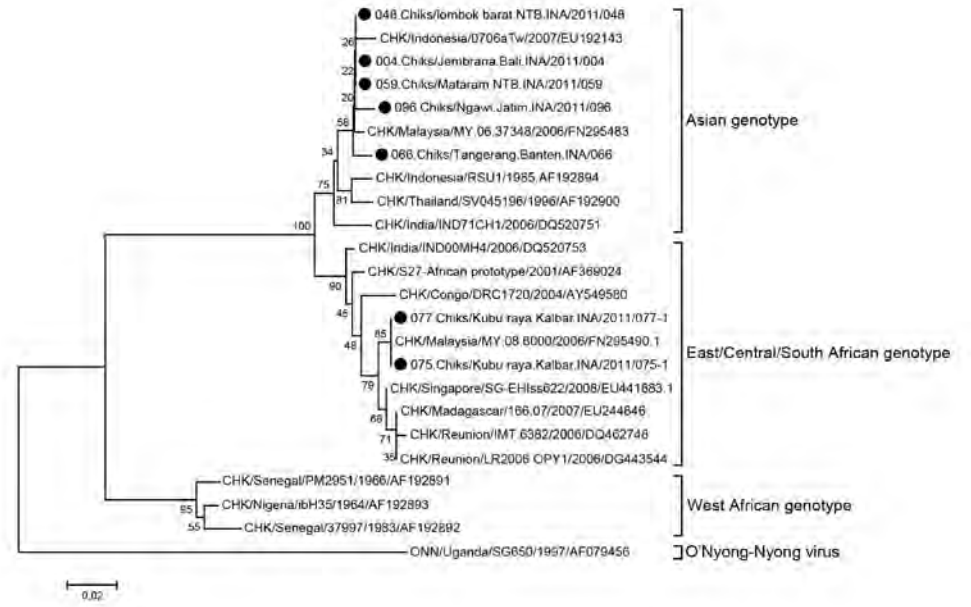

Figure. Cladogram of chikungunya virus, Asian and East/Central/South African genotypes, from chikungunya fever outbreaks in 6 districts in Indonesia, April–October 2011. Black dots indicate samples from patients. Scale bar indicates nucleotide substitutions per site. CHK, chikungunya; Chiks, chikungunya.

Indonesia, although this suggestion is by no means certain. The predominance of the Asian genotypes suggests endemicity of similar CHIKV strains.

A limitation of this study was the lack of serologic assays to confirm CHIKV infections, especially for those who sought care late after onset of illness. Because of the lack of reliable serologic assays with high sensitivity, some cases deemed by RT-PCR to be CHIKV negative might have been clinical cases of CHIKV infection (*10*).

Thus, the A226V ECSA genotype of CHIKV was circulating in Indonesia 5 years after the global pandemic and 2–3 years after the emergence of this strain from other Southeast Asian countries (*4*). Sensitive serology-based assays and rapid tests for different operational settings are needed, especially in those areas without molecular diagnostic capabilities. Also needed is surveillance of CHIKV throughout Indonesia so that health policy makers can have comprehensive data on the molecular epidemiology and prevalence of CHIKV infection. In addition, studies of CHIKV transmission by different vectors as well as virus and vector interactions are needed to provide an understanding of the emergence of the mutant strain across the region and to assist strategies for vector control and disease prevention and control.

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Co-infection with Zika and Dengue Viruses in 2 Patients, New Caledonia, 2014

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To the Editor: Dengue is the most prevalent arthropod-borne viral disease in tropical and subtropical countries. Every year, dengue virus (DENV) infections cause more than 50 million cases, 500,000 hospitalizations, and 12,500 deaths worldwide (*1*). DENV belongs to the genus *Flavivirus* and is transmitted by *Aedes* spp. mosquitoes. There are 4 distinct serotypes (DENV-1 to DENV-4), and infection with 1 serotype does not provide long-term, cross-protective immunity against the other 3 serotypes.

In New Caledonia, DENV outbreaks have occurred since World War II and have been caused mainly by 1 serotype/genotype introduced from a country to which dengue is hyperendemic. Since 2000, New Caledonia has had recurrent DENV-1 outbreaks (*2*). In 2014, circulation of DENV-1 and DENV-3 was still reported in this country (*3*).

Zika virus (ZIKV) is an emerging mosquito-borne virus that belongs to the genus *Flavivirus* and was first isolated in Uganda (*4*). ZIKV is believed to be transmitted to humans by *Aedes* spp. mosquitoes. Before 2007, few human cases of infection had been reported. In 2007, the first Zika epidemic occurred in Yap, Federated States of Micronesia (*5*). In October 2013, a ZIKV outbreak was reported in French Polynesia (*6*).

In New Caledonia, the first cases of ZIKV infection imported from French Polynesia were confirmed at the end of November 2013, and the first autochthonous cases were reported by mid-January 2014. Early in February 2014, the New Caledonia Health Authority declared an outbreak situation. Since February 2014, a total of 1,385 ZIKV laboratory-confirmed cases have been detected, including 35 imported cases (32 from French Polynesia, 2 from Vanuatu, and 1 from the Cook Islands). Concomitant with this ZIKV outbreak, circulation of DENV-1 and DENV-3 was reported; >150 cases were biologically confirmed during January–September 2014 (*3*). Thus, New Caledonia currently has 3 arboviruses co-circulating.

In recent years, co-circulation of multiple DENV serotypes or DENV and chikungunya virus has been reported. Although rare co-infections have been identified (*7*,*8*), given the similar clinical features and lack of concurrent testing, co-infections might not be identified. We report detection of ZIKV and DENV genomes in serum of a traveler (patient 1) who returned from French Polynesia where ZIKV and DENV were co-circulating (*6*,*9*) and in serum of a person (patient 2) in New Caledonia who had no travel history. The traveler was co-infected with ZIKV and DENV-3, and the local patient was co-infected with ZIKV and DENV-1.

Patient 1 was a 14-year-old boy who had fever (39.5°C), headache, arthralgia, asthenia, and myalgia. No hemorrhagic or neurologic findings were reported, and the patient recovered within 3 days. A complete blood count showed mild thrombocytopenia (platelet count 129 × 10^9 platelets/L; reference range 150–400 × 10^9 platelets/L), leukopenia (leukocyte count 2.75 × 10^9 cells/L; reference range 4–10 × 10^9 cells/L) with associated stimulated lymphocytes, and discreet hepatic cytolysis (aspartate aminotransferase 55 IU/L; reference value <34 UI/L, and alanine aminotransferase 51 IU/L; reference value <55 IU/L). Serum was analyzed by using real-time reverse transcription PCR (RT-PCR) and was positive for ZIKV as recommended by Lanciotti et al. (*5*) and DENV-3.

Patient 2 was a 38-year-old woman who had fever (40°C), headache, arthralgia, asthenia, myalgia, retroorbital pain, conjunctivitis, diarrhea, nausea, and a diffuse pruritic maculopapular rash. No hemorrhagic or neurologic findings were reported, but signs of illness lasted ≈1 week. The patient had mild thrombocytopenia (platelet count 123 × 10^9 platelets/L) and leukopenia (leukocyte count 2.65 × 10^9 cells/L). Serum was analyzed by using RT-PCR (*5*) and was positive for ZIKV and DENV-1.

Co-infections were assessed by sequencing partial ZIKV membrane–envelope gene regions for isolates (GenBank accession nos. KM212963 and KM212967) from both patients, partial DENV-1 envelope gene for an isolate (KM212960) from patient 2, and partial DENV-3 nonstructural protein 5 gene for an isolate (KM212962) from patient 1. Sequencing was conducted at La Plateforme du Vivant (Noumea, New Caledonia).

DENV-1 sequence obtained belonged to genotype I and clustered with DENV-1 sequences isolated in New Caledonia (*2*). DENV-3 sequence obtained belonged to genotype III, similar to DENV-3 recently isolated in French Polynesia (*9*). ZIKV sequences obtained belonged to the

Asian lineage and had 99% identity with sequences of ZIKV isolated in French Polynesia in 2013 (*10*).

Serum specimens from both patients were cultured on Vero cells, and supernatants were evaluated by RT-PCR. Each specimen was positive only for DENV, which was probably caused by low viral loads for ZIKV.

We report co-infection of 2 patients with DENV and ZIKV; each patient was infected with a different DENV serotype. No synergistic effects of the 2 viral infections were observed because both patients were not hospitalized and recovered after a mild clinical course.

During this outbreak, patients in New Caledonia were tested for DENV, chikungunya virus, and ZIKV within the framework of the arboviruses sentinel network, which enabled detection of co-infections. Thus, clinicians should be aware of infections with multiple pathogens in the differential diagnosis of dengue-like illness, especially in patients who returned from tropical regions. This diagnostic procedure could be improved by using multiplex RT-PCR for travelers, given the frequent co-circulation of multiple arboviruses in tropical regions.

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Fatal Meningoencephalitis in Child and Isolation of *Naegleria fowleri* from Hot Springs in Costa Rica

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To the Editor: Primary amebic meningoencephalitis (PAM) is an acute and fulminant disease caused by *Naegleria fowleri*, an amphizoic ameba belonging to the family *Vahlkampfidae*. About 235 PAM cases have been described worldwide, most in children and immunocompetent young adults (*1*,*2*). The infection occurs through the nose; the ameba enters through the nasal passages and ascends the olfactory nerve until it reaches the olfactory bulb of the central nervous system. The incubation period for PAM ranges from 5 to 7 days, and infection leads to death within a week. Symptom onset is abrupt, with bifrontal or bitemporal headaches, fever, and stiff neck, followed by nausea, vomiting, irritability, and fatigue. The mortality rate is as high as 95%; few cases of survival have been reported (*2*,*3*).

The epidemiology of most reported cases of PAM indicates an association between aquatic activity and infection. Swimming, free diving, and immersion in hot springs, spas, and warm, freshwater bodies have been related to the acquisition of *N. fowleri* amebae (*3*). To date, most cases have been reported from subtropical or temperate zones, and underreporting in tropical regions has been cited (*2*); in the Americas, PAM has been reported in Venezuela, Brazil, Cuba, Mexico, and the United States. Most infections occur after swimming in water naturally heated by the sun or geothermal water (*2*).

On July 29, 2014, an 11-year old boy, a resident of Florida, USA, was admitted to a hospital for an illness that began after he returned from vacation in La Fortuna, San Carlos, Costa Rica. The infection was fulminant, and the boy died <72 hours after admission. Tests conducted by the hospital confirmed PAM (*4*). The background of the case indicates that the boy spent 1 week in Costa Rica and stayed for 4 days in La Fortuna area. The onset of symptoms occurred 3 days after he left Costa Rica, which is consistent with the incubation period for PAM. Furthermore, the boy's family stated that in Florida they did not allow him to swim in lakes or rivers because of the known risk of amebal infection (*4*,*5*), which further suggests that the infection may have occurred in Costa Rica.

The Florida Department of Health was alerted about the case, and personnel from the Centers for Disease Control and Prevention contacted the Costa Rica Ministry of Health to identify the potential source of infection. Water samples from a swimming pool, a river pond, and a hot spring from the resort visited by the boy in La Fortuna were collected and analyzed within 12 hours. The samples were filtered through nitrocellulose membranes with 0.45-μm pore diameter, and the filters were placed over 1.5% non-nutritive agar plates, supplemented with *Escherichia coli* (*6*). Plates were incubated at 35°C for 7 days and observed daily. After 3–4 days of incubation, cysts and trophozoites with morphologic characteristics compatible to *Naegleria* spp. were observed in the samples from the hot spring and the river pond. Cysts were round and 10–12 μm in size, with a *Limax*-type nucleus. Trophozoites showed very active movement, with wide pseudopods of rapid formation. Results of an exflagellation test were positive, and a thermotolerance test showed organism growth at 44°C –45°C (*7*).

To molecularly characterize the isolate at the species level, we extracted DNA from the culture using the method described by Reyes-Battle et al. (*8*). A specific PCR for *N. fowleri* was performed, and the complete internal transcribed spacer region was amplified as previously described (*1*). The 18S rDNA gene of this free-living ameba was also amplified by using the universal eukaryotic P2 and P3r primer pair (*9*). *N. fowleri* Lee ATCC 30894 DNA was used a positive control in the PCR reactions. The obtained PCR products were purified and sequenced by using a MEGABACE 1000 Automatic Sequencer (Healthcare Biosciences, Barcelona, Spain) in the University of La Laguna Sequencing Service (SEGAI, University of La Laguna). Sequences were obtained twice from both strands and aligned by using MEGA 5.0 software (*10*). Moreover, nucleotide similarity search was performed by BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) of the sequenced amplicons against ameba species. These analyses revealed 97%–98% homology with other *N. fowleri* strains available in GenBank. The sequence isolated in this case has been deposited in GenBank (accession no. KM658156).

In summary, this investigation identified an *N. fowleri* ameba in water sources at a resort in Costa Rica that had been visited by a child from the United States who died of PAM as a results of *N. fowleri* infection. These amebas pose a high risk to human health and were found in an area frequented by tourists, which should alert health authorities in Costa Rica of the need for monitoring locations such as this for possible contamination and notifying the public of the risk for infection.

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Genome Sequence of Enterovirus D68 and Clinical Disease, Thailand

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To the Editor: Outbreaks of respiratory enterovirus D68 infection were particularly severe in 2014 in the United States. Wylie et al. recently analyzed the whole genomes of clinical strains from St. Louis, Missouri, USA, and the US Centers for Disease Control and Prevention (Atlanta, GA, USA) (*1*). Results showed that the most closely related genomes to the St. Louis strains were strains CU134 (GenBank accession no. KM361523) and CU171 (KM361524), which were identified in Thailand in 2011 (*2*,*3*).

To provide additional background regarding the origin of these strains from Thailand, including 1 additional CU70 strain (KM361525), we report clinical features of the 3 patients from which the strains were derived. This additional information might assist clinical scientists in early recognition of enterovirus D68 infections and provide insight into viral pathogenesis.

The 3 patients (1 boy and 2 girls; age range 7–24 months) were hospitalized during July–September 2011 with pneumonia. At admission, they had cough, rhinorrhea, and dyspnea. Fever, crepitation, and wheezing were observed in patients CU70 and CU134. Patients CU134 and CU171 had suprasternal and subcostal retraction, and patient CU171 had signs of nasal flaring and inspiratory stridor (he has an underlying double aortic arch). Chest radiographs showed perihilar infiltration for patients CU70 and CU134. Hemocultures and test results for respiratory viruses for all 3 patients were negative (*2*).

Physicians provided respiratory support to all 3 patients by oxygen flow and nebulized bronchodilator. In addition, patient CU171 was given nebulized adrenaline, an intravenous corticosteroid, and intravenous antimicrobial drugs. Patients CU70 and CU134 were discharged after 3 and 8 days, respectively. However, patient CU171 remained hospitalized for 16 days.

Nasopharyngeal aspirates obtained from the 3 patients were subjected to next-generation sequencing and genomic analysis. From the total number of analyzed reads for isolates from patients CU70 (n = 10,482), CU134 (n = 11,504), and CU171 (n = 4,545), ≈1,100–1,600 enterovirus D68 sequence reads were identified. Anellovirus sequences (n < 60) were found in aspirates from patients CU70 and CU171. Furthermore, aspirates from patients CU134 and CU171 contained human rhinovirus B (n = 73) and human rhinovirus C (n = 15), respectively (*2*). Future genomic studies and surveillance of enterovirus D68 will be helpful in monitoring its spread next season.

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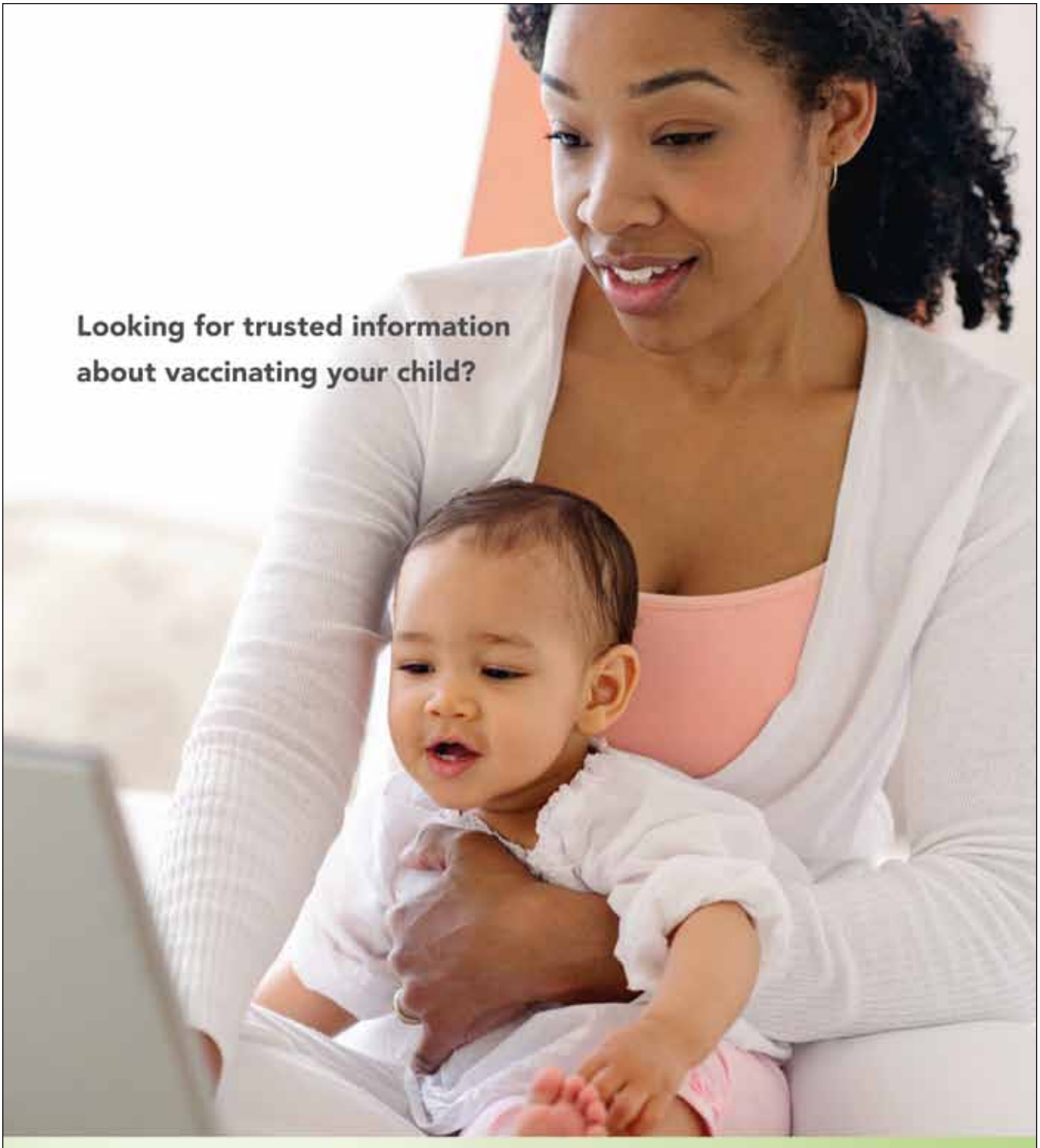
Correction: Vol. 20, No. 11

The name of author Anne-Marie Roque-Afonso was listed incorrectly in the article Foodborne Transmission of Hepatitis E Virus from Raw Pork Liver Sausage, France (C. Renouet al.). The article has been corrected online (http://wwwnc.cdc.gov/eid/article/20/11/14-0791_article.htm).



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Cubism and Research Synthesis

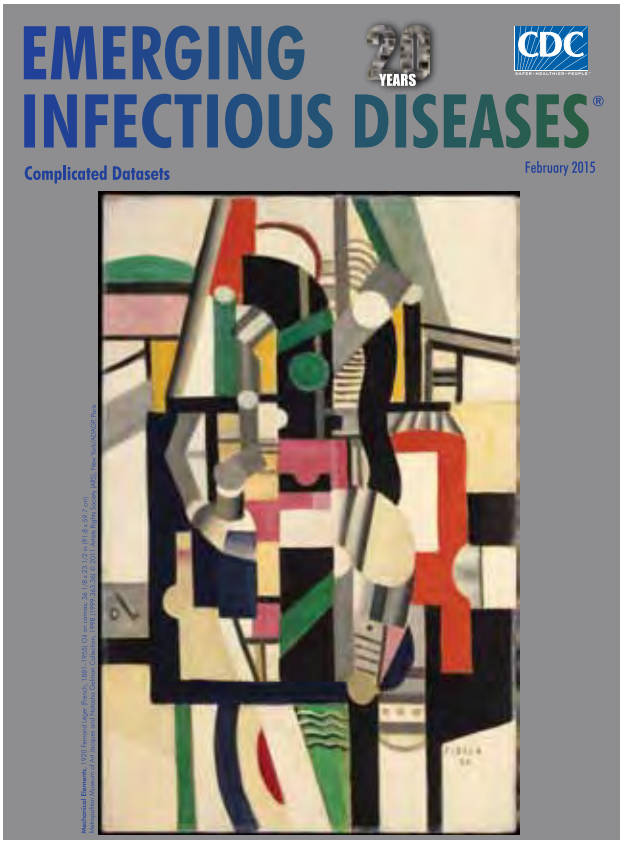
Salaam Semaan

French artist Fernand Léger (1881–1955) embraced the Cubist art movement of fracturing objects into geometric shapes. The term cubism draws on the art critic Louis Vauxcelles' reference to the bizarre cubes he saw in budding artwork that fragmented form into interlocking blocks. Many Cubists reduced objects into cylinders, spheres, and cones and painted them in a single plane as if all faces of an object are visible simultaneously or successively. Influenced by his background as an architectural draftsman and by modernism, Léger was interested in the relationship between color and architecture, perhaps to express the noise, dynamism, speed, and movement of new technology and machinery.

Léger's unique brand of cubism was distinguished by his focus on geometric forms, use of brilliant primary colors, bold black outlines, and belief that everyone could understand art. Léger adapted cubism techniques to break down forms into tubular shapes. His predominant style in 1910 was nicknamed "Tubism."

Cubism can evoke in our minds the methodology of research synthesis conducted via systematic reviews and meta-analysis. Synthesis of clinical or public health research, conducted by combining results of several studies, explores the relationship between an intervention or an exposure and a health outcome from perspectives different from and in addition to those examined in a single study. In cubism, the artist paints an object from different or successive angles as a single image on a single canvas. In research synthesis, clinical and public health professionals examine the scientific literature on a certain topic from multiple years and perspectives. Meta-analysts display the literature in one publication in different figures, including forest plots, funnel plots, and chronological cumulative meta-analysis.

Cubist artwork creates a feeling of movement and shape that can differ from what an object represents. Research synthesis allows for examining scientific studies of diagnostic tools, therapies, interventions, programs, or policies as part of a continuum, with the past flowing into the present and the present influencing the future. By revolutionizing the way objects are depicted and painted, Cubists created a dialogue among art critics, collectors, dealers, and the general public. Research synthesis creates



Fernand Léger (French, 1881–1955)
Mechanical Elements, 1920
Oil on canvas 36 1/8 x 23 1/2 in (91.8 x 59.7 cm)
Metropolitan Museum of Art
Jacques and Natasha Gelman Collection, 1998 (1999.363.36)

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a dialogue among clinical and public health professionals about its role in evidence-based medicine and public health. Set against a framework of thick, black horizontal and vertical lines, Léger's *Mechanical Elements* is characterized by cones, cylinders, disks, and parallel wavy lines that can evoke the image of a new machine age and its momentum or a forest plot and the data of many research studies. In their syncopated arrangement, new technologies transform the world. In a synthesized and pooled effect size, meta-analysis provides new implications for research, programs, and policy.

Learning how to conduct and apply systematic reviews and meta-analysis may be similar to learning how to use machinery. One must learn concepts (e.g., elements of research synthesis, the technology), repeat the behaviors (e.g., use relevant software, operate the machine), and practice relentlessly. Just as art can help the public understand history, clinical and public health professionals can use research synthesis as a scientific tool to enhance medicine and public health. To promote evidence-based programs and policies, the primary single-study articles and research synthesis published in this journal can remind us that we can learn from art and science to conduct, value, and apply evidence-based medicine and public health.

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2015

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EIS
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http://www.cdc.gov/eis/conference.html

May 14–17, 2015
SHEA
The Society for Healthcare Epidemiology of America
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http://shea2015.org/attendees/registration/

May 30–June 2, 2015
American Society for Microbiology General Meeting
New Orleans, LA, USA
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August 24–26, 2015
ICEID
International Conference on Emerging Infectious Diseases
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August 29–September 2, 2015
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20th Annual Infectious Disease Board Review Course
McLean, VA, USA
http://smhs.gwu.edu/cehp/activities/courses/idbr

2016

March 2–5, 2016
ISID
17th International Congress on Infectious Diseases
Hyderabad, India

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

pH Level as a Marker for Predicting Death among Patients with *Vibrio vulnificus* Infection, South Korea, 2000–2011

CME Questions

1. You are seeing a 50-year-old man with a 2-day history of vomiting and fever. Blistering skin lesions have now developed in this patient, and you suspect that he might have an infection with *Vibrio vulnificus*. Which of the following statements characterizes the cohort of patients with *V. vulnificus* in the current study?

A. The cohort was equally divided between women and men
B. Less than 10% of patients had a history of eating raw seafood
C. The majority of patients had a history of potential exposure to *V. vulnificus*
D. Chronic liver disease was rare overall

2. Which of the following statements regarding outcomes of patients with *V. vulnificus* infection in the current study is most accurate?

A. Nearly half of patients died
B. The median time between hospital arrival and time of death was 75 hours
C. Initial application of appropriate antibiotics dramatically improved survival
D. Early surgery dramatically improved survival

3. You want a quick measure of prognosis for this critically ill patient. Which of the following variables was most significantly associated with survival from infection with *V. vulnificus* on multivariate analysis in the current study?

A. Partial pressure of oxygen
B. Serum creatinine
C. Serum aminotransferase levels
D. Active partial thromboplastin time

4. The patient's ABGA results return. What was the optimal cutoff value for pH on ABGA in predicting mortality in the current study?

A. 7.0
B. 7.15
C. 7.35
D. 7.8

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Infectious Causes of Encephalitis and Meningoencephalitis in Thailand, 2003–2005

CME Questions

1. You are seeing a 16-year-old girl with a 3-day history of fever, severe headache, and confusion. She has just returned from travel to Thailand 2 days ago. Encephalitis is in your differential diagnosis. In the current study, what was the most common finding on ancillary studies among patients with encephalitis?

A. Cerebrospinal fluid (CSF) pleocytosis
B. Abnormal result on brain magnetic resonance imaging
C. Abnormal result on brain computed tomography scan
D. Electroencephalographic result consistent with encephalitis

2. As you evaluate this patient, what should you consider regarding the demographic and clinical backgrounds of patients with encephalitis in the current study?

A. Median age was 31 years
B. Nearly all patients were admitted to the hospital during summer months
C. Almost all patients were from rural areas
D. A minority of patients had a substantive co-morbid illness

3. The patient is admitted to the hospital, and you await test results to confirm the etiology of her encephalitis. What percentage of patients in the current study had a confirmed or probable etiology of encephalitis diagnosed?

A. 36%
B. 59%
C. 82%
D. 95%

4. What was the most commonly identified organism responsible for causing encephalitis in the current study?

A. West Nile virus
B. Japanese encephalitis virus
C. Chikungunya
D. *Bartonella henselae*

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
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Strongly Disagree				Strongly Agree
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Ticks and Lyme Disease

For more information about Lyme disease, visit http://www.cdc.gov/Lyme

How to prevent tick bites when hiking and camping

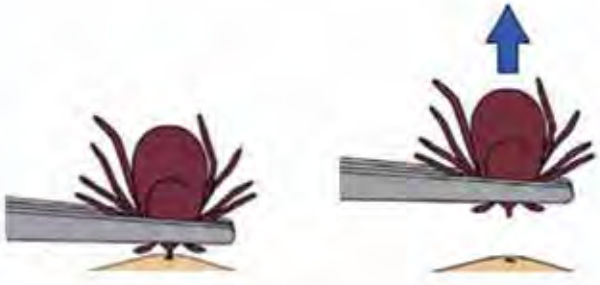
Ticks can spread disease, including Lyme disease. Protect yourself:

- Use insect repellent that contains 20 - 30% DEET.
- Wear clothing that has been treated with permethrin.
- Take a shower as soon as you can after coming indoors.
- Look for ticks on your body. Ticks can hide under the armpits, behind the knees, in the hair, and in the groin.
- Put your clothes in the dryer on high heat for 60 minutes to kill any remaining ticks.

How to remove a tick

1. If a tick is attached to you, use fine-tipped tweezers to grasp the tick at the surface of your skin.
2. Pull the tick straight up and out. Don't twist or jerk the tick—this can cause the mouth parts to break off and stay in the skin. If this happens, remove the mouth parts with tweezers if you can. If not, leave them alone and let your skin heal.
3. Clean the bite and your hands with rubbing alcohol, an iodine scrub, or soap and water.
4. You may get a small bump or redness that goes away in 1-2 days, like a mosquito bite. This is not a sign that you have Lyme disease.

Note: *Do not put hot matches, nail polish, or petroleum jelly on the tick to try to make it pull away from your skin.*



If you remove a tick quickly (within 24 hours) you can greatly reduce your chances of getting Lyme disease.

National Center for Emerging and Zoonotic Infectious Diseases
Division of Vector Borne Diseases | Bacterial Diseases Branch

CS232422D

CDC PROVIDES INFORMATION ABOUT MRSA SKIN INFECTIONS.

Visit **www.cdc.gov/MRSA** or call **1-800-CDC-INFO** to order provider materials including:

- > Clinician guidelines
- > Evaluation & treatment recommendations
- > Patient education materials
- > Posters
- > Fact sheets
- > Flyers

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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.

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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.

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.