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Gustave Doré (1832-1883) *Les Mendiants de Burgos*, 1875. 64 × 119 cm, 25.2 × 46.85 inches
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On the Cover

Gustave Doré (1832–1883)
Les Mendiants de Burgos, 1875

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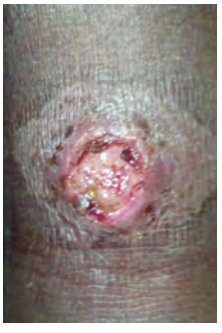

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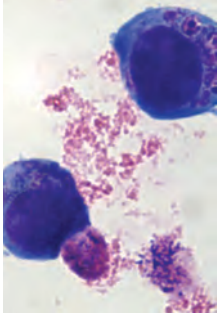

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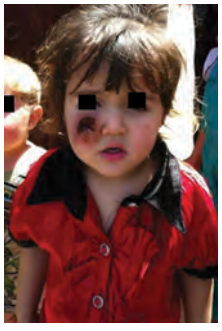

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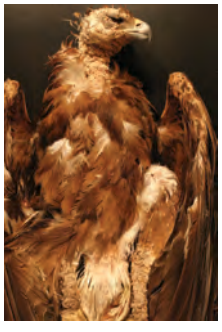

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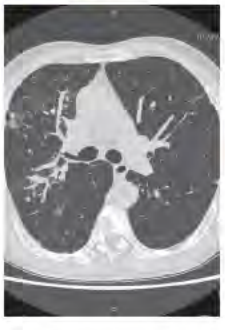

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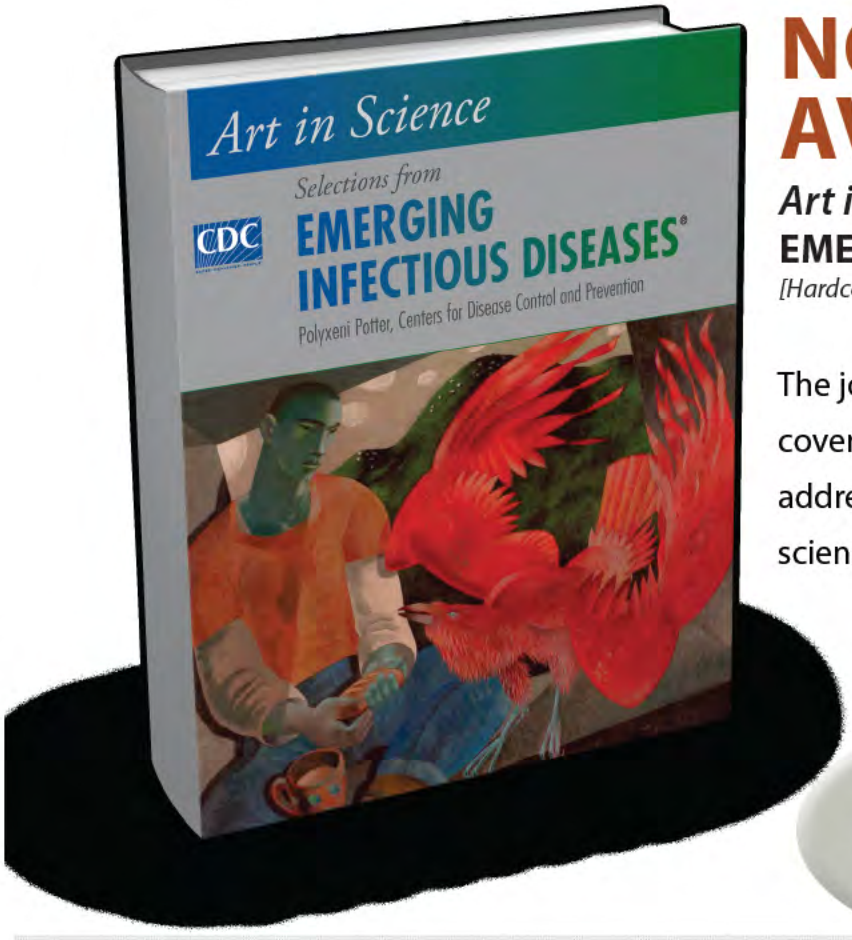

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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Resurgence of Cutaneous Leishmaniasis in Israel, 2001–2012

Dan Gandacu,[1] Yael Glazer,[1] Emilia Anis, Isabella Karakis, Bruce Warshavsky, Paul Slater, and Itamar Grotto

Cutaneous leishmaniasis has long been endemic in Israel. After a 15-year period of moderate illness rates, reported incidence increased from 0.4 cases per 100,000 population in 2001 to 4.4 cases per 100,000 population in 2012, and the disease emerged in areas where its presence had previously been minimal. We analyzed all cases reported to the national surveillance system and found that outbreak patterns revealed an expansion of *Leishmania major* infections over large areas in the southern part of the country and the occurrence of spatially focused *L. tropica* outbreaks in the northern part of the country. Outbreaks often followed new construction in populated areas. Further study of factors affecting the transmission of cutaneous leishmaniasis is needed in Israel, as well as the development of effective methods to control the disease, an increase in awareness among health care professionals, and intensive public education regarding control measures in areas of known leishmaniasis foci.

Leishmaniasis is a disease caused by parasites of the genus *Leishmania* (Trypanosomatidae: Kinetplastida); global incidence approaches 2 million cases annually (*1*,*2*). Cutaneous leishmaniasis (CL), the most common form of the disease (*3*), is endemic in most Mediterranean countries (*4*). Humans become hosts of the disease when the parasitic infection develops in the immune system and causes skin lesions. The lesions tend to heal spontaneously after 3–18 months (*5*) but often result in disfiguring scars (*6*,*7*). Functional complications are rare (*8*).

CL has long been endemic in Israel, and the disease is known colloquially in the region as the "Rose of Jericho." Historically, the main source of the disease in Israel has been *L. major* parasites; cases resulting from this species have been widely distributed in the Negev region in the Southern health district, the arid and semi-arid area of southern Israel that that is sparsely populated and accounts for ≈60% of the country's land. More recently, illness caused by *L. tropica* parasites has been reported in several semi-arid hilly areas in Israel's more densely populated, and less dispersed, central and northern population centers (*9*,*10*).

The recognized vector of CL in Israel is the female phlebotomine sand fly. The species predominantly responsible for CL cases in Israel have been *Phlebotomus papatasi* for *L. major* infections and *Ph. sergenti* for *L. tropica* infections. The reservoir of *L. major* parasites consists of rodents (e.g., *Psamomys obeesus*, *Meriones crassus*, *Microtus guentheri*, *Meriones tristrami*, *Gerbillus* spp.), whereas the main reservoir of *L. tropica* parasites is the rock hyrax (*Procavia capensis*). *L. tropica* infection tends regionally to be an urban, anthroponotic phenomenon, but in Israel it is zoonotic in nature and has an incubation period that is longer than that for *L. major* infection and is more resistant to treatment. *L. tropica* infection also results in multiple lesions and, when it results in leishmaniasis recidivans, has a lower tendency to heal spontaneously (*11*,*12*).

Before 2001, a total of 3,352 cases of CL had been reported in Israel, and annual incidence ranged from 0.1 to 7.3/100,000 population. Two periods of particularly high illness rates occurred during this time, in 1967–1969 and in 1980–1982 (*13*). The first peak, in the late 1960s, appeared after the June 1967 War, after the exposure of naive populations to the parasite in disease-endemic areas. The second peak, in the early 1980s, was assumed to have resulted primarily from the continuing increase in the number of new settlements westward from the Jordan Valley toward Jerusalem (*13*). A decade and a half of relatively moderate incidence followed, and at the end of 2000, reported national incidence stood at 0.3/100,000 population. However, by 2012, reported incidence had increased to 4.4/100,000 population and CL had emerged in areas where its presence had previously been minimal. We describe these changes from an epidemiologic point of view and discuss factors

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[1]These authors contributed equally to this article.

that might explain the increase in CL rates and distribution from 2001 to 2012.

Methods

The Israeli Ministry of Health has conducted routine national surveillance of CL since 1949, when the disease became reportable. Cases are reported through a computerized notification system network from each of 15 regional health districts in the country. The ministry's Division of Epidemiology (DOE) monitors and processes the case report data on a national basis. Although the surveillance method is passive and is assumed to underestimate the actual number of CL cases, no changes in reporting methods and no official efforts to increase the frequency of notification have been implemented. Therefore, we assumed in our research that the reported incidence data are a good indication of actual epidemiologic trends in the population.

Two caveats should be noted regarding any CL incidence data. Underreporting of CL is widely acknowledged to be a problem for a variety of clinical, logistic, cultural, and other reasons (*14*); we discuss some of the underlying causes for underreporting later in this article. However, during major outbreaks or when infection occurs in previously non–CL-endemic areas, heightened awareness may increase the proportion of CL cases that are officially reported.

Laboratory confirmation of suspected CL cases is currently not required, and therefore, laboratory tests are not routinely performed on samples from all reported case-patients. Consequently, both probable and confirmed CL cases are reported to the DOE, and distinctions cannot be made in the national incidence data between *L. major* and *L. tropica* infections. Because the laboratory results that are available provide an indication of the *Leishmania* species, these results can be used to make reasonable species-related assumptions regarding specific outbreaks and regarding other reported cases in nearby geographic areas where another species has not been detected. However, for those areas in which >1 species of *Leishmania* is found, such assumptions cannot be made (*15*).

We analyzed all CL cases that were reported to the Israel national surveillance system for which onset occurred during January 1, 2001–December 31, 2012. For methodologic reasons, all cases reported here are from the civilian population; the Israeli military uses active surveillance for CL and commonly includes only cases with a clinical diagnosis.

The denominators used for calculations of crude and specific incidence rates per 100,000 persons according to age, sex, population group, and health district were derived from annual population estimates from the Central Bureau of Statistics of Israel (*16*). We used χ^2 tests for comparison between rates. All statistical analyses were conducted by using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and SPSS version 19.0 (SPSS IBM, Armonk, NY, USA). We considered $p<0.05$ as statistically significant.

Results

Overall, 2,061 cases of CL that occurred in Israel were reported during January 1, 2001–December 31, 2012. Of the case-patients, 58% were men, and 93.8% were Jewish. The mean age at disease onset was 31.1 ± 22.1 (range 0–90, median 28) years. More than 42% of reported cases occurred during 2010–2012, and 40% of case-patients were residents of the Southern health district/Negev region.

From 2001 to 2012, the CL incidence rate in Israel increased tenfold, from 0.4 to 4.4/100,000 population (Figure 1). Over this period, mean annual incidence increased by 0.36/100,000 population. These increases reflect, in part, the spread of CL to central and northern areas of the country, where reports of the disease had previously been minimal. Those increases were highlighted by several outbreaks and incidence peaks. An outbreak occurred in the Kinneret subdistrict near Lake Tiberias in northern Israel in 2003; several dozen cases were reported, sometimes >1 per family, in 2 new periphery neighborhoods at the edge of the city of Tiberias. During 2004–2005, an outbreak of >100 cases occurred in 2 new neighborhoods near the ravines of a suburb, Ma'ale Adumim, in the Jerusalem health district. A peak in national incidence occurred in 2007–2008, a result of an increase in CL cases in the Southern health district, where *L. major* parasites are endemic, and an outbreak of *L. tropica* infections in the northern district. Whereas cases from the southern region were sporadically distributed, in the northern region, cases were restricted to peripheral houses near the borders of 1 rural settlement in the Yizre'el subdistrict, where few cases had been reported in the past. The main sources of a subsequent peak in 2012 were the occurrence of 37 cases in Ofakim, a small town in the Southern health district, and 44 cases that occurred with the continuation of the outbreak in Ma'ale Adumim (24.2% and 64.7% of the district totals, respectively). Toward the end of 2012, a new outbreak began in Carmiel, in the Akko subdistrict. CL illness rates also increased within the Southern health district in several localities, including Be'er Sheva, Eilat, Yeruham, and Arad, and expanded elsewhere to other, much smaller, rural villages (Figure 2).

Among age groups, increases in incidence were highest during most years among infants. Nevertheless, the 15–44-year-old age group comprised the greatest portion of case-patients each year compared with the other age groups; these differences were significant in 2006 ($p = 0.03$), 2007 ($p = 0.016$), 2009 ($p = 0.025$), 2010 ($p<0.001$), and 2012 ($p<0.001$) (Table). CL incidence was generally higher among the male population than among the female population, except for those ≥65 years of age.

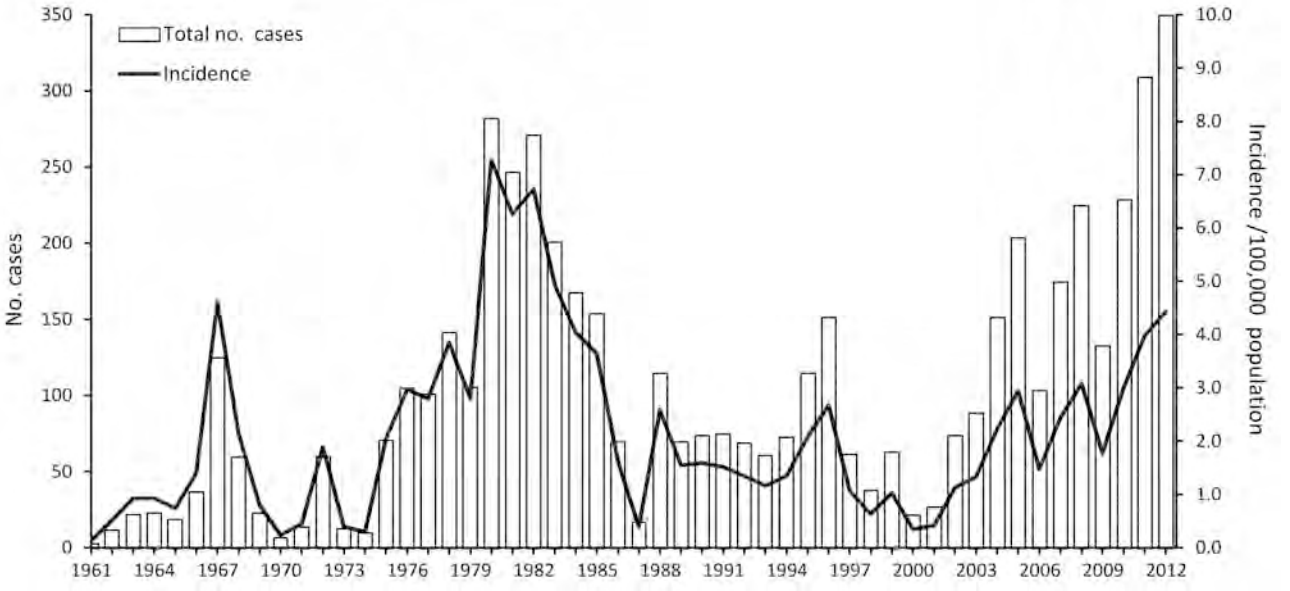

Figure 1. Cutaneous leishmaniasis in Israel, 1961–2012, showing annual number of cases and incidence per 100,000 population. A sharp increase is shown for the study period, 2001–2012.

Reports of CL during 2001–2012 were markedly dissimilar by population group. More than 93.8% of reported case-patients were Jewish (range 81.5%–94.4%), even though Jews made up only 75%–77% of the national population during the study period (*17*). Similarly, annual incidence rates for Jews were consistently higher than those for non-Jews ($p = 0.032$). The mean incidence rate for Jews was 2.9/100,000 (range 0.4–5.4) versus 0.6/100,000 (range 0.1–1.3) for non-Jews (Figure 3).

Discussion

Trends in reported CL in Israel during 2001–2012 indicate an increase in the incidence of the disease, particularly in recent years, and the spread of CL to new areas. Regional patterns in reported incidence rates show numerous outbreaks of CL spreading beyond the Southern health district, which had traditionally been the source of most cases in Israel, to regions of the country in which reports of the disease had previously been minimal. Whereas CL in the south is mostly endemic and occurs sporadically over large areas (the sporadic nature of *L. major* infection has also been noted in nearby countries [*18*]), the outbreaks in the north have been more focused both temporally and spatially. In the 2003 outbreak in Tiberias, for example, CL cases appeared within a short time span and often infected family clusters. An *L. major* outbreak that occurred in the eastern area of the Yizre'el subdistrict (Beit She'an valley) was similarly focused and distinct from outbreaks in southern areas in Israel to which *L. major* parasites are endemic. In the latter outbreaks, reported cases typically had no epidemiologic linkage in time or place and occurred in such localities as Yeruham, Dimona, Ofakim, and various rural villages that are widely dispersed throughout the southern region of the country.

Many of the CL outbreaks in the northern region of Israel were notable for the emergence of *L. tropica* parasites, which had not previously been known to be a common cause of CL in Israel. The increasing incidence of *L. tropica* infections has also been observed in other countries in the Mediterranean Basin and elsewhere in Asia (*19*,*20*,*21*). In addition to the resulting increase in CL incidence, the presence of *L. tropica* parasites poses other concerns. Infections caused by this species tend to be more diffuse and difficult to treat than infections caused by *L. major* parasites and can sometimes lead to more chronic or more dangerous forms of the disease, such as leishmaniasis recidivans or, in rare cases, visceral leishmaniasis.

The incidence of CL has been increasing globally during the past decade (*22*). Research into the reasons for this occurrence has identified human activity and interventions in the environment (e.g., migration, urbanization, and deforestation [*23*–*26*]), longer-term or natural events (e.g., climate change [*27*] and earthquakes [*28*]), and improvements in the detection and diagnosis of the disease. In Israel, the increase in CL incidence rates several decades ago was explained by immigration of naive populations into endemic areas, but today, this reason is applicable only to persons from non–CL-endemic areas who visit CL-endemic regions. The most probable causes for increased incidence in more recent years are the changes to the vector-reservoir-human population network resulting from land development and construction, the expansion of populated

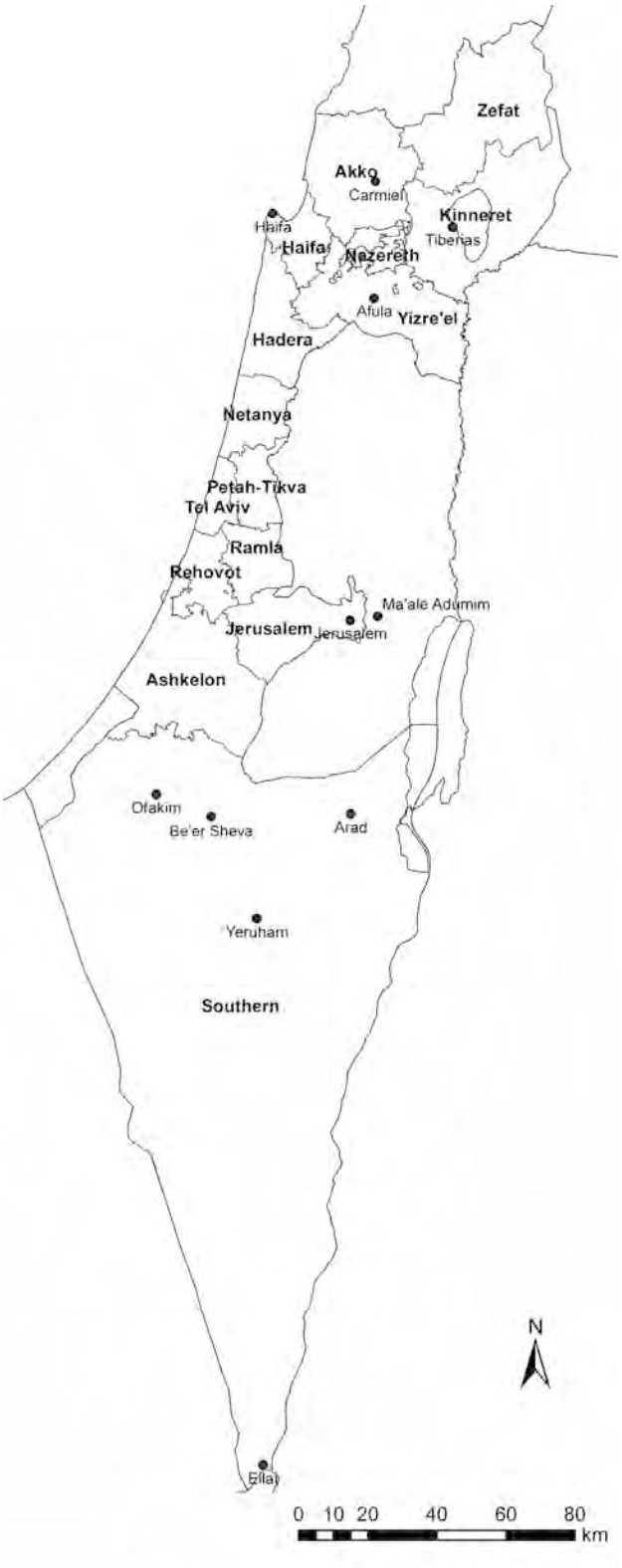

Figure 2. Selected localities (black dots) where cases of cutaneous leishmaniasis were reported in Israel during 2001–2012. Health districts are labeled in boldface.

areas, and changes in land use (such as in Tiberias, Ma'ale Adumim, and Ofakim). These factors have altered the relationships of human communities with the surrounding natural environment and have created favorable conditions for mammalian reservoirs and sand fly populations to breed in close proximity to human habitations (*23*,*24*), a phenomenon that has also occurred elsewhere (*29*). This combination of events has facilitated transmission of CL in established populated areas among persons who had lived there for many years, as well as the spread of the parasite to new locations.

Climate change may also contribute to the spread of CL by influencing (e.g., through increased temperatures or rainfall) variables such as vector life cycles, mammalian reservoir abundance, disease transmission patterns, and the geographic scope of the pathogen (*30*,*31*). Preliminary research has shown a high correlation between temperature increases and the incidence of human CL in Israel (*32*).

Examination of monthly reporting patterns over the past several decades showed a shift in the seasonal peak of CL case reports from the summer to the autumn. This change in seasonality may reflect the population dynamics of the sand fly vector for *L. tropica* parasites (*33*–*35*) compared with the vector for *L. major* parasites. Further study is needed to clarify whether the continuing emergence of *L. tropica* parasites in the northern region of Israel is partly responsible for the seasonality shift or whether other causative factors are involved.

Reported CL incidence in Israel has consistently been higher among the male population, except among persons ≥65 years of age, which correlates with findings from studies in other CL-endemic countries in which the relationship between sex, age, and CL has been addressed (*29*,*36*). Similarly, the total number of reported CL cases in Israel is highest among adults of working age, as has also been found elsewhere (*37*). However, in almost every year during the study period, the CL incidence rate for infants was greater than for any other group, which may reflect diagnoses made during routine well-baby care examinations that are provided at Israel's Mother and Child clinics. If so, this finding may be a cause of information bias (diagnostic bias) in the case surveillance system, a reflection of the fact that infants do not have immunity to CL, as may be the case with children and adults. Because of data limitations in the reporting method used in this study, the age and sex attributes could not be tested for confounders or effect modifiers that could result from other factors, such as lifestyle, working place, time spent outdoors during sand fly activity hours, and insect repellent usage.

A notable difference continues to appear in the CL reporting rates among the country's major population groups. The reported incidence rates for the non-Jewish population were fairly constant and were lower during the study period

Table. Annual cutaneous leishmaniasis incidence and proportion of total cases, by age group, Israel, 2001–2012

Year	Incidence/100,000 population, by age group, y					Proportion of total cases, by age group, y				
	<1	1–14	15–44	45–64	≥65	<1	1–14	15–44	45–64	≥65
2001	0.0	0.6	0.4	0.3	0.3	0.00	0.37	0.41	0.15	0.07
2002	2.2	1.3	0.9	1.4	1.1	0.04	0.30	0.34	0.23	0.09
2003	2.1	1.4	1.3	1.6	0.6	0.03	0.28	0.42	0.22	0.04
2004	4.9	2.0	2.6	1.9	1.0	0.05	0.24	0.51	0.16	0.05
2005	2.1	3.5	2.3	3.2	3.9	0.01	0.31	0.34	0.20	0.13
2006	4.1	1.9	1.2	1.4	1.0	0.06	0.35	0.36	0.17	0.07
2007	5.4	2.7	2.4	3.0	0.3	0.05	0.29	0.42	0.23	0.01
2008	3.9	3.7	2.8	3.6	1.5	0.03	0.32	0.39	0.22	0.05
2009	2.5	2.5	1.5	1.6	1.1	0.03	0.37	0.37	0.17	0.06
2010	4.9	3.1	2.3	4.5	2.5	0.03	0.27	0.32	0.29	0.08
2011	6.7	3.5	3.7	5.0	3.4	0.04	0.23	0.39	0.24	0.09
2012	1.8	4.0	3.8	5.8	4.7	0.01	0.24	0.37	0.26	0.11

than those for the Jewish population. Because of the limitation of the passive surveillance reporting system, it is difficult to know exactly how much of this difference reflects actual incidence and how much results from other factors, such as the influx of non–CL-immune Jews into existing disease foci or the preference in some non-Jewish communities in CL-endemic areas not to seek treatment for a familiar skin lesion. The difference may also be explained in part by other cultural and behavioral traditions that can influence health-seeking behavior, particularly because the gap in incidence has remained constant over the years. In some CL-endemic areas in the Middle East and southern Asia, for example, the appearance of permanent scarring after CL infection may lead to social consequences such as shame, stigmatizing effects, or harm to a woman's chances for marriage (*38*,*39*). Because CL infection provides immunity from future infection by the same *Leishmania* species, a common folk custom among some population groups is to proactively acquire infection with the parasite to generate natural immunity against future disease. This process involves deliberately exposing infants, especially girls, to the bite of a phlebotomine sand fly on a part of the body that is normally covered by clothing (*40*). This practice may continue to some extent in Israel among some desert-dwelling Bedouin; however, we believe the practice has become far less prevalent in Israel among this population subgroup because of continuing trends toward modernization and urbanization.

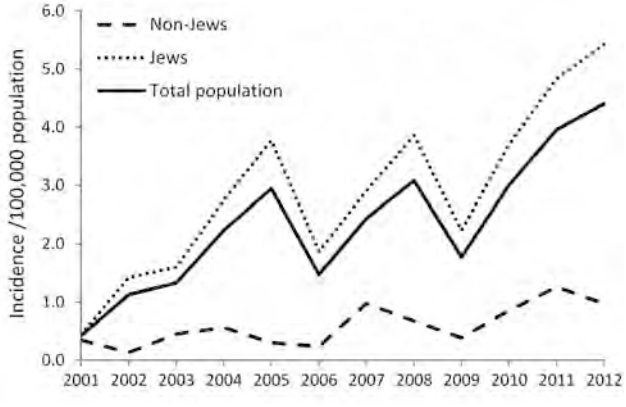

Figure 3. Annual incidence of cutaneous leishmaniasis by population group (Jews vs. non-Jews), Israel, 2001–2012.

Assuming that the underreporting of actual illness was fairly constant during the years of study, the incidence data we present show cyclical trends that reasonably reflect the 2 main currents of human CL in Israel during the 2001–2012 period: 1) the sporadic CL that has long been endemic to southern Israel; and 2) the occurrence of focused CL outbreaks resulting from population movement, land development, and the emergence of *L. tropica* parasites in other areas of the country. Although passive surveillance does not permit a more accurate estimation of the full extent of CL disease in Israel, the reported incidence data clearly show an expansion of CL to areas in which CL was not previously known to be endemic. The concern that reported CL cases not only have been increasing in volume but also are emerging in geographic areas where illnesses previously had been rare has led an inter-ministerial committee to institute a National Program to Reduce Leishmaniasis Hazards in Israel. This 3-year program, led by the Ministry of Environmental Protection in collaboration with the Ministries of Health, Defense, and Interior, and the Nature and Parks Authority, includes the experimental implementation of different intervention methods designed to reduce disease transmission and increase personal protection in 50 localities, as well as the initiation of scientific and feasibility studies of relevant zoonotic, epidemiologic, and control issues.

In summary, a review of nationwide outbreaks over a recent 13-year period has revealed an expansion of CL illness in southern, central, and northern areas of Israel. Regional differences were seen regarding the dominant causative species for infection and the geographic and temporal patterns of outbreaks. Further study of the factors affecting CL transmission is needed, as are the following: development of effective methods to control CL infection, an increase in health professionals' awareness of the disease, and a greater degree of public alertness. Intensive public education regarding control measures in areas of known leishmaniasis foci is required.

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Dr Gandacu is a public health physician in the Division of Epidemiology of the Israel Ministry of Health. His research interest is the epidemiology of zoonotic diseases.

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Rapidly Growing Mycobacteria Associated with Laparoscopic Gastric Banding, Australia, 2005–2011

Hugh L. Wright, Rachel M. Thomson, Alistair B. Reid, Robyn Carter, Paul B. Bartley, Peter Newton, and Christopher Coulter

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Learning Objectives

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

- Describe the mode and characteristics of transmission of mycobacterial infections associated with laparoscopic gastric banding, based on a case series
- Discuss clinical features and complications of mycobacterial infections associated with laparoscopic gastric banding
- Assess management of mycobacterial infections associated with laparoscopic gastric banding

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Laparoscopic gastric banding is a common bariatric procedure worldwide. Rapidly growing mycobacteria are environmental organisms increasingly seen as pathogens, often in infected prosthetic material. We report 18 cases of infection associated with laparoscopic gastric banding caused by *Mycobacterium fortuitum* and *M. abscessus* in Australia during 2005–2011. We identified cases by reviewing positive cultures at the Queensland state reference laboratory or through correspondence with clinicians, and we obtained clinical and epidemiologic data. Eleven cases of *M. fortuitum* and 7 cases of *M. abscessus* infection were identified. The port was thought to be the primary site of infection in 10 of these cases. Complications included peritonitis, band erosion, and chronic ulceration at the port site. Rapidly growing mycobacteria can infect both port and band and can occur as either an early perioperative or late infection. Combination antimicrobial therapy is used on the basis of in vitro susceptibilities. Device removal seems to be vital to successful therapy.

The exponential increase in obesity and morbid obesity worldwide has led to a corresponding increase in bariatric surgical procedures to prevent obesity-associated illness and death (*1*). Laparoscopic gastric banding is a restrictive procedure involving insertion of an inflatable silicon band at the gastric cardia near the gastro-esophageal junction, which enables adjustment of the size of the outlet through the addition or removal of aqueous solution through a subcutaneous port in the abdominal wall. It is the most common bariatric procedure performed in Australia and the United Kingdom (*2*); perceived advantages include its less technical surgical demands and low rates of perioperative complications (*3*). More than 11,000 procedures were performed in Australia during 2011 (*4*). Infection rates are reportedly low (*3*) but can occur at the site of the subcutaneous port or be associated with the band itself.

Rapidly growing mycobacteria are ubiquitous organisms found in environmental sources, including soil and water. They cause skin and soft tissue infections and pulmonary disease but also have a predilection for causing diseases involving implanted prosthetic material. Infections associated with silicone implants, indwelling intravenous or peritoneal catheters, cardiac devices, and prosthetic joints have been reported (*5*–*8*). Isolated cases of mycobacterial infection involving gastric banding have been reported in recent years (*9*,*10*). We report 18 cases of rapidly growing mycobacterial infections associated with laparoscopic gastric banding in Australia during 2005–2011.

Methods

We identified cases by a variety of methods. We systematically reviewed positive cultures for rapidly growing mycobacteria isolated at Queensland Mycobacterium Reference Laboratory (QMRL, Brisbane, Queensland, Australia) that were associated with laparoscopic gastric banding based on the clinical notes provided with the specimen. Other cases were identified by direct clinical involvement by the authors. Additional cases were identified through correspondence with infectious diseases physicians and microbiologists within Australia. Approval was obtained from the local area human research ethics committee associated with the reference laboratory. Clinical and microbiological data for each case, including band manufacturer, technique, and timing of access of the port, as well as the solution used, and treatment records where available were obtained from the treating surgeons, physicians, or microbiologists involved and by examining the hospital medical record.

Organisms were speciated in mycobacterium reference laboratories after referral from laboratories where primary isolation occurred. QMRL characterized 14 isolates using phenotypic and molecular methods, including the Genotype Mycobacteria CM line probe assay (Hain Lifesciences, Nehren, Germany).

Five patients were seen within a narrow temporal period within 9 months of each other, with cultures that isolated *M. fortuitum*. These isolates were further investigated by using pulsed-field gel electrophoresis (PFGE) and repetitive sequence–based PCR (rep-PCR) strain typing using the Diversilab system (bioMérieux, Melbourne, Victoria, Australia) to exclude clonality and delineate the possibility of a point source as the cause of these infections. Because of the wide geographic diversity of cases and delay between infections and outbreak recognition, environmental sampling around the 18 patients was not possible. However, the isolates received were compared with stored environmental isolates from another study (*11*) and other clinical isolates received by QMRL.

For the rep-PCR method, DNA was extracted from 10 clinical isolates by using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA). The PCR mixture was prepared by using AmpliTaq polymerase and PCR buffer (Applied Biosystems, Hammonton, NJ, USA) and Mycobacterium DiversiLab primer mix according to the manufacturer's instructions (bioMérieux). Rep-PCR products were separated and detected by microfluidic chips of the Diversilab System. Fingerprints were analyzed with Diversilab software v.3.4.38 by using the Pearson correlation coefficient and unweighted pair group method with arithmetic means to compare isolates and determine clonal relationship. PFGE was performed on the same 10 clinical isolates and results compared with the patterns generated by automated rep-PCR. Based on the Tenover (*12*) classification of isolates using PFGE, the Diversilab rep-PCR similarity cutoffs were determined as >97% (indistinguishable), >95% (similar), and <95% (different).

PFGE was performed by using the method outlined in the BioRad Genpath Group 6 Kit (BioRad, Marnes-la-Coquette France) with modifications outlined by Mazurek et al. (*13*) and Burki et al. (*14*). Organisms were inoculated into 10 mL Middlebrook 7H9 broth (Difco, Becton Dickinson, Sparks, MD, USA, in-house media) supplemented with 0.2% OADC (Difco, Becton Dickinson), 0.1% Tween 80 (MP Biochemicals, Solon, OH, USA), cycloserine (1 mg/mL; Sigma-Aldrich, St Louis, MO, USA), and ampicillin (0.1 mg/mL; Sigma-Aldrich) and incubated for 3 d. One milliliter of broth was centrifuged and the supernatant discarded.

Gel plugs were prepared and incubated in 500 μL of lysis buffer 1 and 20 μL Lysozyme (25 mg/mL) at 36°C. After a wash step, 500 mL buffer and 20 mL Proteinase K (>600 U/mL) were added to each sample. Plugs were incubated for 48 h at 50°C. The plugs were then washed 4 times in 1× wash buffer. After the final wash, the plugs were stored in 1× wash buffer. Digestion was performed by using *Xba*1 enzyme (10 U/mL), and the samples were incubated for 18 h at 36°C.

The plugs were loaded into wells of a 1% PFGE agarose gel (BioRad), ensuring that no air bubbles formed. Sufficient 0.5× tris-borate-EDTA was added to the PFGE cell and cooled to 14°C, and electrophoresis was performed by using the following parameters: Initial A time 1 s, Final A time 40 s, voltage 200 V, and time 22 h. After electrophoresis, the gel was stained by using ethidium bromide (BioRad), de-stained in running distilled water for 30 min, and then photographed. Antimicrobial susceptibility testing was performed at QMRL by using broth microdilution in accordance with the Clinical and Laboratory Standards Institute guidelines (*15*).

Results

We identified 18 cases of rapidly growing mycobacterial infections associated with adjustable gastric bands over a 6-year period; the causative organism was *M. fortuitum* in 11 of these patients and *M. abscessus* in 7. Mean age of patients was 45 years; 15 (83%) patients were female (Table 1). The average weight of patients was 133 kg at time of insertion of laparoscopic adjustable gastric band. In 5 patients, diabetes mellitus previously had been diagnosed. No patients had been treated with glucocorticoids or other immunosuppressant medications. Time between initial insertion of device and infection varied widely; 8 (44%) cases occurred within the first 3 months (range 21 days–8 years) after insertion. Ten patients initially had primary port site infection; 3 patients had a concurrent port site infection and abdominal symptoms; and 5 patients had abdominal symptoms alone that suggested primary band infection. The most common symptoms associated with band infection were fever, abdominal pain, nausea, and vomiting. Three patients sought care within 4 weeks after band insertion because of evidence of associated microperforation or erosion of the gastric lumen around the band site endoscopically or intra-operatively. Cultures were often polymicrobial with rapidly growing mycobacteria isolated in the presence of *Staphylococcus* spp., enteric gram-negative organisms, or *Candida albicans*. Of the 8 patients who had primary band involvement or features consistent with combined band/port involvement, 5 sought care within 3 months after insertion.

Table 1. Demographic and clinical characteristics of 18 patients with rapidly growing mycobacterial infections complicating laparoscopic gastric band devices, Australia, 2005–2011

Characteristic	Value
Age, mean (range), y	45 (29–64)
M:F	3:15
Weight, mean, kg	133
Co-morbidities, no. (%)	
Diabetes mellitus	5 (28)
Hypertension	6 (33)
Obstructive sleep apnea	4 (22)
Depression	4 (22)
Immunosuppression/glucocorticoid use	0
Causative organism, no. (%)	
M. fortuitum	11 (61)
M. abscessus	7 (39)
Primary site of infection, no. (%)	
Port	10 (56)
Band	5 (28)
Combined port/band	3 (16)
Time from insertion to presentation, no. (%)	
Early, <3 mo	8 (44)
Late, >3 mo	10 (56)
Associated complications, no.	
Peritonitis	2
Erosion/perforation	5
Chronic ulcer	2
Antimicrobial drug therapy	
Median duration (range), mo	6 (3–12)
Combination therapy, no. (%)	17 (94)

Patients who had infection at the primary port site commonly had more indolent signs and symptoms. Pain and erythema at the site were commonly reported. Most of these patients received initial empiric therapy for common bacterial skin and soft tissue pathogens before the causative organism was identified.

Complications included granulomatous peritonitis in 2 patients for whom *M. abscessus* was confirmed on peritoneal biopsy. Infection associated with erosion at the band site occurring >1 month after insertion occurred in 2 additional cases. In 3 patients with primary port site infections, chronic ulcers developed at the port site after device removal; mean time to resolution of ulcer was 9 months, and *M. abscessus* was the causative agent in 2 of these cases.

PFGE and strain typing by using Diversilab platform on 5 *M. fortuitum* isolates showed sufficient genetic diversity to exclude clonality (Figure 1). Gastric bands in this series were inserted atdifferent centers; given the temporal

and geographic diversity of cases, no single point source was identified. In cases where the ports had been accessed, all clinicians reported use of sterile saline or sterile water as the solution for band inflation, performed using sterile techniques by the surgeons themselves.

The strain of *M. abscessus* isolated from 1 of the patients with primary band infection was indistinguishable from an environmental isolate recovered from a suburban rainwater tank. Another patient with *M. abscessus* infection had a strain that differed from the environmental water isolates recovered from another study (Figure 2). The *M. fortuitum* isolates that were available for strain typing differed from those isolated from municipal water (Figure 3) and from other clinical isolates associated with both community and nosocomial infections (Figure 4).

No clear temporal relationship was identified between access of the port and development of port site infection. No cases were reported to have developed within 4 weeks after most recent access. In at least 4 cases, time from most recent access to development of symptoms at port site was >1 year.

All patients received empiric antibacterial therapy before isolation of the mycobacterium. In 1 case of granulomatous peritonitis, empiric first-line therapy for tuberculosis was begun pending identification of the causative organism. Seventeen patients initially received combination therapy upon confirmation of growth of rapidly growing mycobacteria. Initial intravenous therapy was administered in 5 of the 11 cases involving *M. fortuitum* and all **7** involving *M. abscessus*. Duration of intravenous therapy ranged from 2 to 6 weeks and was followed by ongoing oral therapy. The agents used intravenously were amikacin (12 cases), cefoxitin (10 cases), and imipenem (2 cases). Combination therapy with ciprofloxacin and trimethoprim/sulfamethoxazole was the most common regimen used for *M. fortuitum* infections (7 of 11 cases) and reflected the susceptibility data for the isolates (Table 2). Other agents used were clarithromycin, doxycycline, and minocycline.

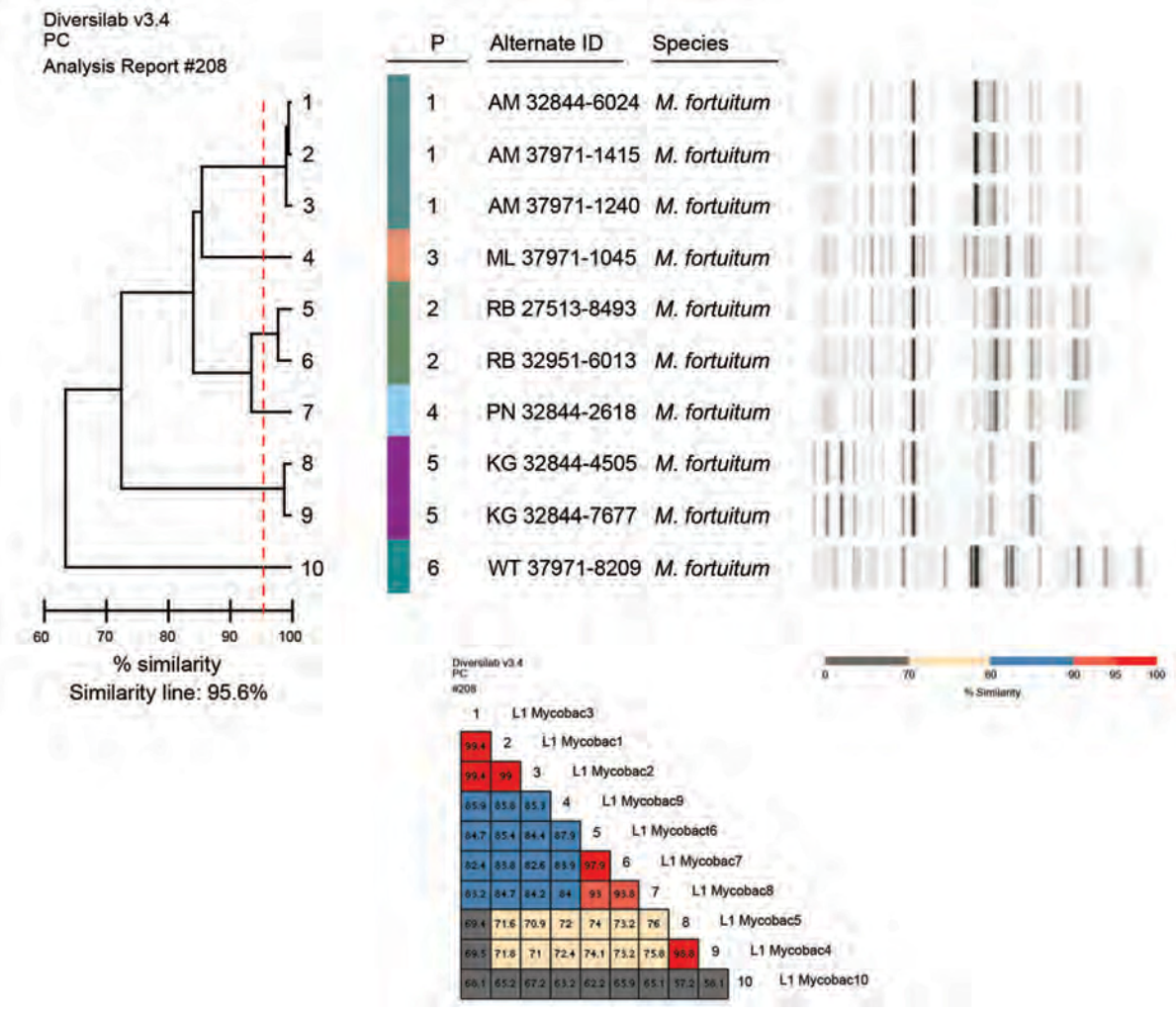

Figure 1. Strain typing using Diversilab platform (bioMérieux, Melbourne, Victoria, Australia) and pulsed-field gel electrophoresis of *Mycobacterium fortuitum* isolates.

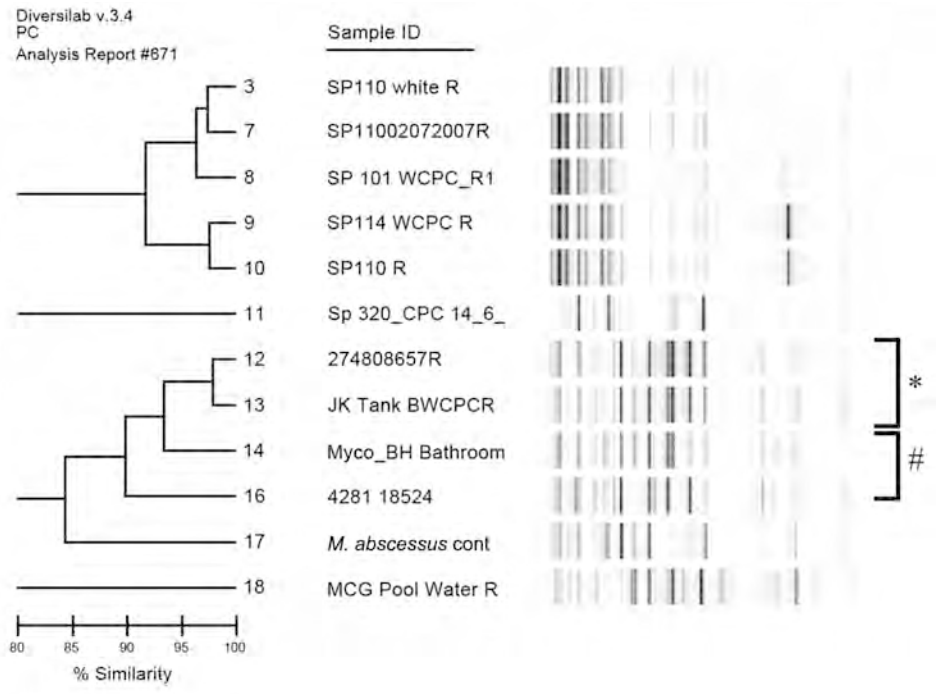

Figure 2. Repetitive sequence–based PCR dendrogram comparing strain types of 2 *Mycobacterium abscessus* isolates associated with laparoscopic band infections, with a laboratory control strain, and 9 other environmental isolates, Australia. *Isolate 12 (patient PB) is indistinguishable from strain 13, isolated from a domestic rainwater tank. #Strain 16 (patient MC) shares 90% similarity with an epidemiologically unrelated domestic bathroom water isolate.

Therapy for the **7** patients with *M. abscessus* infections was more uniform; oral clarithromycin was used in all cases after an initial intensive phase of amikacin and cefoxitin. Total duration of antimicrobial therapy ranged from 3 to 12 months (median 6 months).

In all cases, infection was cured only with complete explantation of the device. In 5 cases for which initial signs and symptoms were consistent with primary port infection, an initial strategy of conservation of the band component was attempted in conjunction with antimycobacterial therapy. In all instances, symptoms subsequently recurred, which resulted in the need for complete removal of the device.

Discussion

Rapidly growing mycobacteria are increasingly recognized as major pathogens, capable of causing a wide spectrum of clinical illness (*16*). Infection from these organisms after surgical procedures, although uncommon, has been well described and is often seen when the procedure involves implantation of prosthetic material (*17*). The mode of acquisition of infection remains unclear in some cases. In cases that occurred shortly after surgery, infection is likely to have been acquired at the time of surgery. *M. fortuitum* and *M. abscessus* have been reported as causes of wound infections from a variety of surgical procedures, including contamination of aqueous solutions or of the surgical equipment used (*18*,*19*).

Minor trauma has been reported as a risk factor for rapidly growing mycobacterial skin and soft tissue infections (*20*). An advantage of laparoscopic gastric banding is the ability to inflate or deflate the band to alter its restrictive effect; however, this procedure might provide a possible portal of entry for infection, particularly in the absence of strict aseptic technique. Alternatively, mycobacterial colonization of the solution used to inflate or deflate the band could result in infection (*21*). We did not find any history of antecedent injury before port site infection, and accessing the port to adjust the band was not associated temporally with port site infection. Although the port and band is a contiguous device, and infection with 1 component appears to lead to involvement of the entire device, the possible pathogenesis of infection might differ depending on the anatomic site at which infection develops primarily. Most cases in which the band was primarily involved were associated with injury to the gastric wall: microperforation or erosion occurred in 5 (63%) of 8 patients. Infection might have been a secondary event that occurred after perforation and subsequent contamination of the band with gastric contents. Alternatively, band infection itself might have factored in damaging gastric integrity.

Devices or implants can become colonized during manufacture because mycobacteria are present within the environment, especially in water sources. Implantation of colonized porcine heart valves has resulted in pericarditis and endocarditis (*22*). We found no evidence to suggest that any cases in our current report resulted from such colonization; devices from different manufacturers were used and the cases were sporadic. Further investigation of cases in which presentation (though not insertion of the gastric band) was temporally related, showed that the isolates differed enough to exclude a point source.

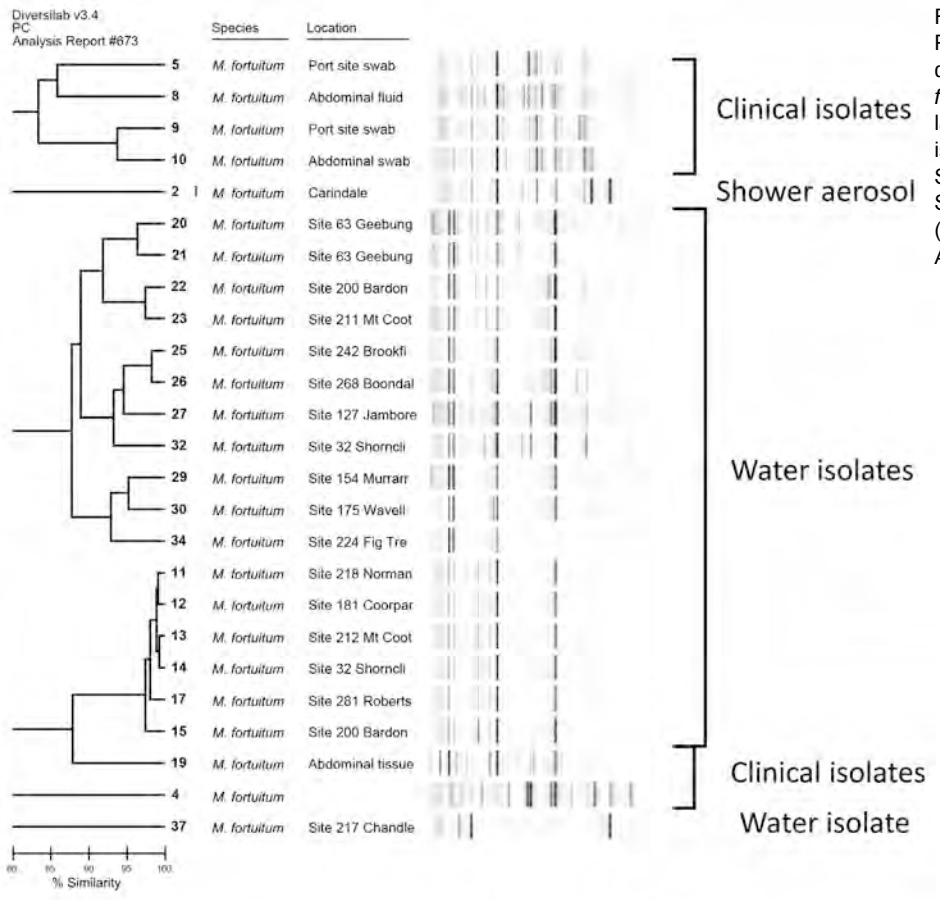

Figure 3. Repetitive sequence–based PCR dendrogram demonstrating differences between *Mycobacterium fortuitum* iso-lates associated with lap band infections and *M. fortuitum* isolated from water samples. Scale bar indicates % similarity. Source: Diversilab v. 3.4 PC #675 (bioMérieux, Melbourne, Victoria, Australia).

Trial data to inform treatment of rapidly growing mycobacteria are lacking. In vitro susceptibilities vary between species, although resistance to first-line antituberculosis agents is common in *M. fortuitum* and *M. abscessus*. Current guidelines from the American Thoracic Society and the Infectious Diseases Society of America (ATS/IDSA) (*23*) suggest therapy on the basis of susceptibility testing and advocate combination therapy. Macrolides are commonly used and are often the only freely available oral agent with activity against *M. abscessus*, as shown in our report. However, rapidly growing mycobacteria can develop resistance by mutations in the peptidyltransferase region of the 23S ribosome gene (*24*). Furthermore, inducible macrolide resistance has been demonstrated in *M. fortuitum* (*25*) and *M. abscessus* (*26*); thus, monotherapy with this agent is not recommended, even if the isolate appears susceptible. The ATS/IDSA guidelines suggested that treatment for serious soft tissue infections caused by *M. abscessus* consists of clarithromycin, with initial therapy also including amikacin with or without cefoxitin. Suggested treatment for *M. fortuitum* infection is combination therapy with at least 2 active agents as guided by in vitro susceptibilities to prevent development of resistance (*27*). Treatment of the infections reported here is consistent with these guidelines. For infected prosthetic material, as shown here, removal of such material appears to be a critical factor in treatment success and is strongly recommended (*23*). Optimal duration of adjuvant antimicrobial therapy remains elusive and may be influenced by how promptly the device is removed.

Laparoscopic gastric banding is a safe and effective method to enable weight loss in obese patients. It remains the most common bariatric surgery performed in Australia; perioperative death rates are very low (*28*). However, evidence is mounting of increasing rates of long-term complications associated with gastric banding is increasing in comparison with the other common bariatric procedure performed worldwide,

Roux-en-Y gastric bypass (*29*). Late complications reported include higher rates of long-term reoperation, band slippage with pouch dilation, port dislocation, erosions, and infection of port or band. Evidence also exists that roux-en-Y gastric bypass provides greater excess weight loss (*30*), with a greater reduction of obesity-associated co-morbid conditions (*31*).

Our retrospective series has limitations. Case finding relied in part on recollection of the physician or surgeons

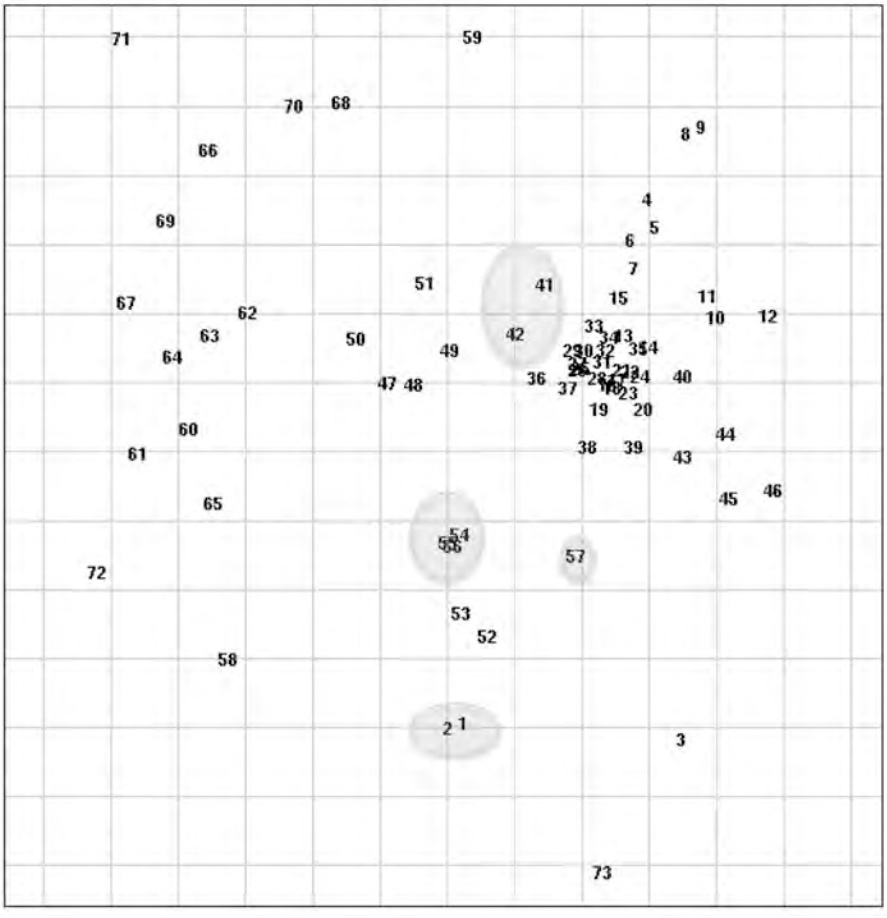

Figure 4. Repetitive sequence–based PCR scatterplot demonstrating lap band isolates (circled) relative to other clinical strains of *Mycobacterium fortuitum* associated with community-acquired and nosocomial infections. Spacing between samples may be distorted if the dataset is large and/or if there is no distinct clustering. Gridline spacing: 5% similarity. Source: Diversilab v. 3.4 PC #675 (bioMérieux, Melbourne, Victoria, Australia).

interviewed. We reviewed positive cultures from the reference laboratory where the rapidly growing mycobacteria were isolated , but because review relied on adequate clinical notes to identify cases associated with gastric banding, some cases might have been missed. We included cases that occurred early and late after device implantation, which might encompass several different etiologic processes. Although we conducted both epidemiologic and molecular investigations, a clear source of infection was not identified. The treatment observed was not standardized as may be attempted in a prospective trial, which may give clearer guidance as to optimal approach.

Table 2. Susceptibilities of *Mycobacterium* spp. isolated in laparoscopic gastric banding to selected antimicrobial agents, Australia, 2005–2011

Antimicrobial agent	*M. fortuitum*, no. (%), n = 11	*M. abscessus*, no. (%), n = 7
Amikacin	10 (91)	7(100)
Cefoxitin	7 (64)	5 (71)
Clarithromycin	3 (27)	7 (100)
Ciprofloxacin	10 (91)	0
Minocycline	2 (18)	0
Imipenem	8 (80)	0
Trimethoprim/sulfamethoxazole	9 (82)	0
Amoxicillin/clavulanate	1 (13)	0

M. fortuitum and *M. abscessus* should be considered as possible etiologic agents of infection associated with laparoscopic gastric banding, arising from port or band. Infection can occur early during the perioperative period or many years after insertion. Prolonged therapy with combination antimicrobial agents is suggested in conjunction with complete removal of the device.

Dr Wright is an infectious diseases physician at the Royal Brisbane and Women's Hospital. His research interests include zoonosis and mycobacteria.

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Distinct Characteristics and Complex Evolution of PEDV Strains, North America, May 2013–February 2014

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Porcine epidemic diarrhea virus (PEDV), which emerged in the United States in 2013, has spread throughout North America. Limited availability of PEDV complete genomes worldwide has impeded our understanding of PEDV introduction into the United States. To determine the relationship between the North American strains and global emerging and historic PEDV strains, we sequenced and analyzed complete genomes of 74 strains from North America; the strains clustered into 2 distinct clades. Compared with the initially reported virulent US PEDV strains, 7 (9.7%) strains from 4 states contained insertions and deletions in the spike gene (S INDELs). These S INDEL strains share 99.8%–100% nt identity with each other and 96.2%–96.7% nt identity with the initial US strains. Furthermore, the S INDEL strains form a distinct cluster within North American clade II, sharing 98.6%–100% nt identity overall. In the United States, the S INDEL and original PEDV strains are cocirculating and could have been introduced simultaneously.

Porcine epidemic diarrhea virus (PEDV) (family *Coronaviridae* family, genus *alphacoronavirus*) has an enveloped, single-stranded, positive-sense RNA genome of ≈28 kb (*1*). The 5′ two thirds of the genome contains 2 large open reading frames (ORFs), 1a and 1b, that encode 2 nonstructural polyproteins, pp1a and pp1b, that direct genome replication and transcription. The remaining PEDV genome contains ORFs specifying structural and nonstructural proteins in the following order: spike (S), ORF 3, envelope (E), membrane (M) and nucleoprotein (N) (*2*,*3*).

Porcine epidemic diarrhea (PED) was first documented in the United Kingdom in 1971 as a swine disease resembling transmissible gastroenteritis (*4*). In 1978, the etiologic agent of PED was identified in Belgium as a new coronavirus and was designated as PEDV, prototype strain CV777 (*1*). Within 2 decades, PEDV was reported in several other European countries; Hungary, Italy, Germany, France, Switzerland, and the Czech Republic (*5*). Currently, the virus causes only isolated outbreaks in Europe. In Asia, PEDV was first identified in 1982 and is now considered endemic, causing substantial economic losses to pork producers in China, South Korea, Thailand, and Vietnam (*5*). However, it was not until 2010 that massive PED outbreaks were reported in China; the outbreaks have been characterized by 80%–100% illness among infected swine herds and a 50%–90% mortality rate among infected suckling piglets (*6*–*8*).

North America was free of PEDV until its sudden and intense emergence in the United States in April 2013 (*9*). Since then, PEDV has spread rapidly across the United States, causing high rates of death among piglets and substantial economic losses (*10*–*12*). As of July 24, 2014, PEDV had been reported in 31 US states (*13*). In Canada, PEDV was first detected in January 2014 on a pig farm in Ontario Province; since then, the virus has been reported on farms in Manitoba, Prince Edward Island, and Quebec Provinces (*14*), continuing its spread throughout North America. There are no official reports of PEDV in Mexico; however, in 2013, the University of Minnesota (UM) Veterinary Diagnostic Laboratory (St. Paul, MN, USA) tested swine samples from Mexico and found them positive for PEDV.

The complete genomic sequence of the PEDV prototype strain, CV777, was determined in 2001 (*15*). A decade later, complete genomes were sequenced for several PEDV strains from China and South Korea (*6*,*8*,*15*–*22*). Comparisons of full-length genomes showed that different PEDV

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strains are more closely related to bat alphacoronaviruses than to other known alphacoronaviruses (*23*), suggesting that interspecies transmission of coronavirus may have occurred decades ago between bats and pigs or through an intermediate host.

Phylogenetic analysis has shown that some PEDV strains that have reported decreased virulence in the field contain distinct insertions and deletions in the S gene (S INDELS). Soon after the emergence of PEDV in the United States, the complete genomes were determined for several strains from Colorado, Minnesota, and Iowa, USA (*10*,*24*,*25*); these PEDV strains shared ≥99.5% nt identity with strain AH2012 from China, suggesting a common ancestor for that strain and US strains (*25*,*26*). An additional 43 complete-genome PEDV sequences are now available: 16 from the United States, including a recent PEDV variant from Ohio (OH851) that contains specific S INDELs and was reported with reduced disease severity (*27*); 23 from China; and 4 from South Korea. In addition, new, complete-genome PEDV sequences are being generated and released almost monthly.

A major impediment to understanding the origin, evolution, and diversity of PEDV in the United States is the lack of complete-genome PEDV sequences worldwide. To determine the phylogenetic relationship between the new US strains and the globally emerging and historic PEDV strains, we sequenced and analyzed the complete genomes of 74 strains from North America.

Materials and Methods

Sample Collection and Processing

Between May 6, 2013, and February 28, 2014, porcine intestine, saliva, and feces samples and fecal swab and environment samples (N = 25,762) from North America were submitted to the UM Veterinary Diagnostic Laboratory for detection of PEDV. RNA was extracted from the samples and screened for PEDV by using previously described methods (*28*,*29*).

Sequencing and Genome Assembly

A total of 74 PEDV-positive samples (72 from the United States, 2 from Mexico) were randomly selected for complete-genome sequencing. Sequencing and genomic assembly were conducted at the UM Genomic Center, using next-generation sequence technology as previously described (*25*). GenBank accession numbers for the PEDV strains are KJ645635–708.

Phylogenetic Analysis

We aligned PEDV sequences by using the ClustalW (http://www.clustal.org/clustal2/) method. Phylogenetic trees for the complete genome and each gene segment were constructed by using the maximum-likelihood method with the general time reversible nucleotide substitution model and bootstrap tests of 1,000 replicates in MEGA 6.06 software (*30*). The RIP (Recombinant Identification Program; http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was used to identify recombination points within the PEDV genome. The RIP analysis parameters were window size of 400 and confidence threshold of 90%.

Results

Phylogenetic Analysis of the Complete Genomes

A phylogenetic tree was constructed on the basis of a multiple sequence alignment of 112 sequences (the 74 PEDV sequences generated from this study and the 38 PEDV sequences available from GenBank). Most of the PEDV strains from the United States shared complete-genome nucleotide identities of 98.6%–100% and were distributed between 2 clades within tentative genogroup 2a (Figure 1) (*23*). Compared with the initially reported prototype strains from the United States, 7 (9.7%) of the 72 newly identified US strains had an S INDEL. These 7 PEDV strains were from 4 US States, shared 99.8%–100% nt identity with each other and 96.2% nt identity with the prototype North American strains, and formed a separate cluster in clade II. In addition, phylogenetic analysis of the 112 complete PEDV genomes confirmed a recent common ancestor with PEDV strains from China and all PEDV strains from North America, as was suggested previously (*25*,*26*). Because the choice and number of sequences are known to affect tree topology, we also compared the 112 PEDV complete genomes with 11 bat alphacoronavirus complete genomes as out-group sequences (data not shown) to validate the observed phylogeny of the PEDV strains from the United States. This analysis confirmed the closest recent common ancestor with strain AH2012 from China and PEDV strains from the United States, and it confirmed the presence of the 2 North American clades and the S INDEL cluster from the United States. Of the 4 states with S INDEL strains, Minnesota had the lowest detection rate for these strains (6.9%, 2 of 29 strains), followed by Ohio (14%, 1 of 7 strains); the highest rates were in Iowa (43%, 3 of 7 strains) and Indiana (50%, 1 of 2 strains).

North American PEDV clade I and clade II were uneven in size (n = 43 and n = 24, respectively) and had unequal representations from the US states; some states were represented in only 1 clade (Table). The analyzed PEDV strains from Ohio (n = 6) and Illinois (n = 4) belonged to clade I, and the PEDV strains from Oklahoma (n = 3) were grouped within clade II. PEDV strains from Minnesota constituted 42% (18 of 43 strains) of clade I and 37.5% (9 of 24 strains) of clade II, and PEDV strains from Iowa constituted 4.7% (2 of 43 strains) of clade I and 8.3% (2 of 24

strains) of clade II, corresponding with the higher sampling in these 2 states. Two isolates from Mexico, Mexico124 and Mexico104, belonged to clades I and II, respectively.

A comparison of the phylogenetic grouping and temporal distribution of the 74 PEDV strains from North America showed that clade I mostly consisted of strains collected between November and December 2013 (n = 38); only 5 clade I stains were collected during July–October 2013 (Table). In contrast, 1–6 clade II strains were collected each month throughout the year. The S

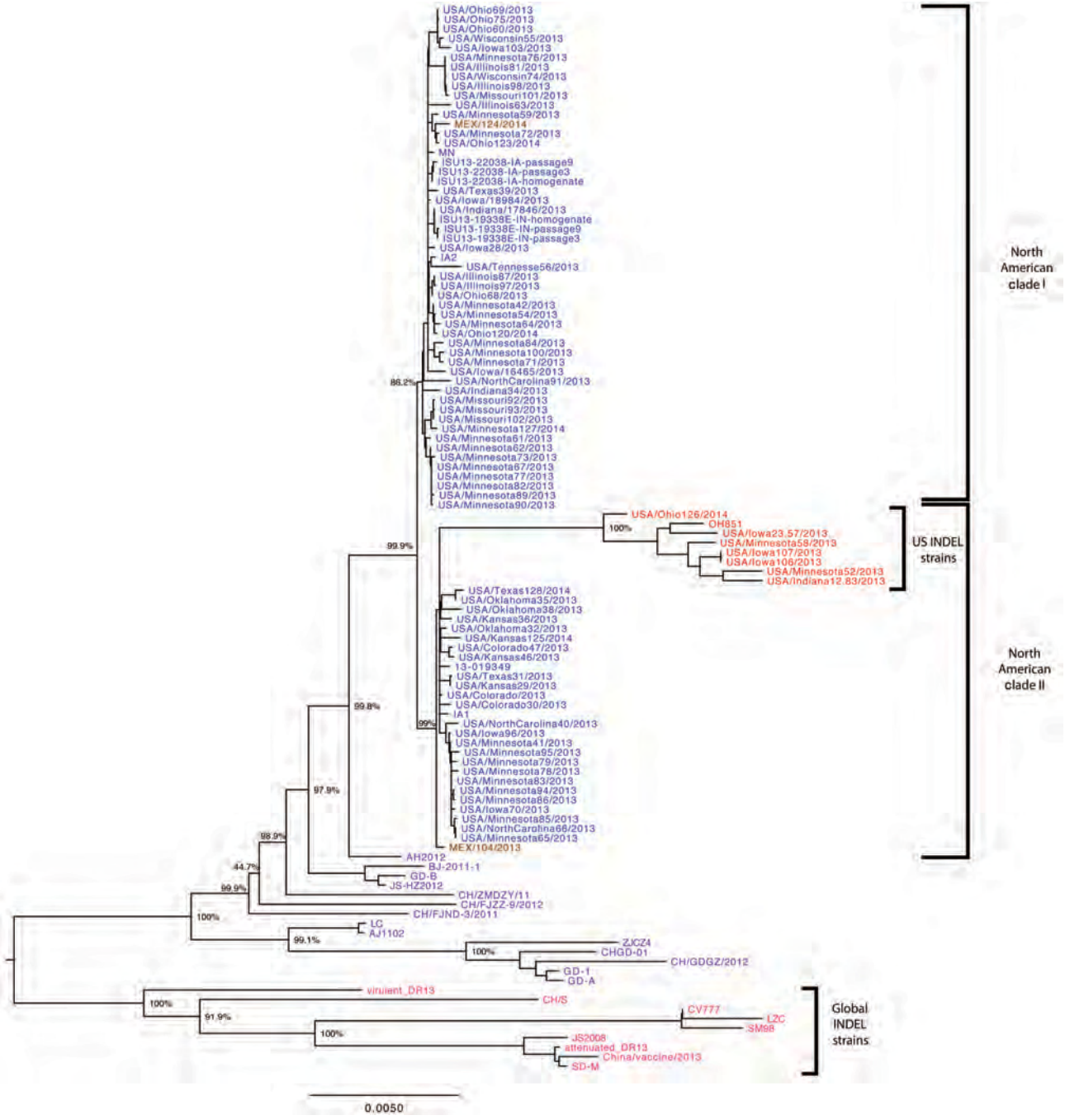

Figure 1. Phylogenetic tree based on complete genome sequences of 112 North American porcine epidemic diarrhea virus strains. Blue represents US non–S INDEL strains; red represents US S INDEL strains; brown represents Mexican strains; purple represents worldwide non–S INDEL strains; and pink represents global S INDEL strains. Bootstrap values are represented at key nodes. Scale bar indicates nucleotide substitutions per site. CH, China; IA, Iowa; S INDEL, insertions and deletions in the spike gene; IN, Indiana; ISU, Iowa State University; MEX, Mexico; MN, Minnesota; USA, United States of America.

INDEL strains from the United States were also detected throughout the year (1–2 strains/month). The first S INDEL strain, USA/Indiana12.83/2013, was detected in feeder pigs on June 5, 2013.

The 38 PEDV strains that were not from the United States or Mexico formed 3 distinct branches corresponding to genogroups 1a, 1b, and 2b/R, as previously reported (*26*). Other than the S INDELs, a comparison of the complete-genome sequences of all 112 PEDV strains did not show any insertions or deletions that were specific for PEDV strains from the United States; non–S INDEL mutations were represented by single-nucleotide polymorphisms (SNPs).

Phylogenetic Analysis of ORF 1 Regions

We also analyzed a phylogenetic tree based on the ORF 1 region of the North American and global PEDV strains (data not shown). We observed 2 identical North American clades that included, as in the complete-genome analysis, all of the strains from the United States and Mexico. The 7 S INDEL strains from the United States grouped together with strain CH/ZMDZY/11 from China but not with strain

Table. Phylogeny and spatiotemporal distribution of porcine epidemic diarrhea virus strains detected in North America, May 2013–February 2014*

Month and year detected	Clade I	Clade II	S INDEL
June 2013			USA/Indiana12.83/2013
July 2013	USA/Iowa28/2013	USA/Kansas29/2013 USA/Colorado30/2013 USA/Texas31/2013 USA/Oklahoma32/2013	
August 2013	USA/Indiana34/2013	USA/Oklahoma35/2013	
September 2013	USA/Texas39/2013	USA/Kansas36/2013 USA/Oklahoma38/2013	
October 2013	USA/NorthCarolina40/2013 USA/Minnesota42/2013	USA/Minnesota41/2013 USA/Kansas46/2013 USA/Colorado47/2013	USA/Iowa23.57/2013
November 2013	USA/Minnesota54/2013 USA/Wisconsin55/2013 USA/Tennesse56/2013 USA/Minnesota59/2013 USA/Ohio60/2013 USA/Minnesota62/2013 USA/Illinois63/2013 USA/Minnesota64/2013 USA/Minnesota67/2013 USA/Ohio68/2013 USA/Ohio69/2013 USA/Minnesota72/2013 USA/Minnesota73/2013 USA/Wisconsin74/2013 USA/Ohio75/2013 USA/Minnesota76/2013 USA/Minnesota77/2013	USA/Minnesota65/2013 USA/NorthCarolina66/2013 USA/Iowa70/2013 USA/Minnesota78/2013 USA/Minnesota79/2013	USA/Minnesota58/2013 USA/Minnesota52/2013
December 2013	USA/Illinois81/2013 USA/Minnesota82/2013 USA/Illinois87/2013 USA/Minnesota89/2013 USA/Minnesota90/2013 USA/NorthCarolina91/2013 USA/Missouri92/2013 USA/Missouri93/2013 USA/Illinois97/2013 USA/Illinois98/2013 USA/Minnesota100/2013 USA/Missouri101/2013 USA/Missouri102/2013 USA/Iowa103/2013 USA/Minnesota127/2013 USA/Minnesota61/2013 USA/Minnesota71/2013 USA/Minnesota84/2013	USA/Minnesota83/2013 USA/Minnesota85/2013 USA/Minnesota86/2013 USA/Minnesota94/2013 USA/Minnesota95/2013 USA/Iowa96/2013 Mexico104/2013	USA/Iowa106/2013 USA/Iowa107/2013
January 2014	USA/Ohio120/2014 USA/Ohio123/2014 Mexico124/2014	USA/Texas128/2014 USA/Kansas125/2014	USA/Ohio126/2014

*S INDEL, insertions and deletions in the spike gene.

AH2012 from China, suggesting that molecular signature characteristics of the S INDEL strains are not limited to the specific S INDELS. However, the tentative parent strain, AH2012, clustered together with the non–S INDEL PEDV strains from North America.

Comparative sequence analysis of the 7 US S INDEL strains showed the presence of 99 specific SNPs in ORF 1b (10 specific to the US S INDEL strains and 89 similar to SNPs in CH/ZMDZY/11) that were not present in the other 80 North American strains. Of the 89 SNPs that were similar to SNPs in CH/ZMDZY, 13 were identical in all 7 S INDEL strains, including USA/Iowa23.57/2013 and USA/Ohio126/2014, which were slightly different from the other 5 S INDEL strains; this finding was also observed in the phylogenetic analysis (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/10/14-0491-Techapp1.pdf). In addition, among 76 of the 89 specific SNPs that were similar to those in CH/ZMDZY/11, 9 were identical in 6 of the 7 S INDEL strains (excluding USA/Iowa23.57/2013 and/or USA/Ohio126/2014), 20 were identical in 5 of the 7 S INDELS strains (excluding USA/Iowa23.57/2013 and USA/Ohio126/2013), and 47 were identical in 6 of the 7 S INDEL strains (excluding USA/Ohio126/2014); however, these 76 specific SNPs were not found in any other analyzed PEDV strains from North America or in strain AH2012 from China.

Phylogenetic Analysis of the S Genes

We conducted S gene phylogenetic analysis on the 112 North American and global PEDV strains (Figure 2). The 7 S INDEL PEDV strains from the United States shared 99.8%–100% nt identity with each other for the entire S gene, and they shared 96.6%–97.1% nt identity with the other 67 PEDV strains from North America. In contrast to findings from the complete-genome analysis, findings from the S gene analysis indicated that strain CH/ZMZDY/11, but not strain AH2012, from China was most closely related to the non–S INDEL PEDV strains from the United States.

Phylogenetic analysis of the S gene did not show the 2 North American clades observed in the complete-genome analysis. Instead, all 112 analyzed PEDV strains were distributed between just 2 major branches, each of which had several minor sublineages. The first branch contained global PEDV sequences lacking the S INDELs; the second branch contained global PEDV S INDEL strains with US and non-US sublineages. Nucleotide sequence comparison showed that these S INDELs (1-nt, 11-nt, and 3-nt deletions at positions 167, 176, and 416, respectively, and a 6-nt insertion between positions 474 and 475) were identical in the US PEDV strains and global S INDEL strains. Moreover, 104 SNPs were identical in the S1 region (in the first 1,400 nt) of the S gene of the US and most global S INDEL strains.

The remainder of the S1 region and the full S2 region of the S gene shared high nucleotide identity among the US and global PEDV strains. Phylogenetic analysis of these regions showed that S INDEL and non–S INDEL PEDV strains from the United States were most closely related to strain CH/ZMZDY/11, but not to strain AH2012, from China (data not shown).

Phylogenetic Analysis of ORF 3 of PEDV Strains from North America

Phylogenetic analysis of ORF 3 did not show any specific differences between the S INDEL and non–S INDEL PEDV strains from the United States (data not shown). All PEDV strains from North America grouped together, sharing 95.4%–95.7% nt identity. ORF 3 was not an immediate target region in the recent evolution of S INDEL strains in the United States. As determined by ORF 3 sequence analysis, the recent CH/ZMZDY/11 and BJ-2011-1 strains from China were most closely related to the strains from North America, and the remaining global PEDV strains formed several branches separate from the North American strains.

Phylogenetic Analysis of E, M, and N Genes

Phylogenetic analyses of the genes for E, M, and N structural proteins showed that all PEDV strains from North America formed a monophyletic branch, sharing 99.5%–100% nt identity, and there were no major differences in these genomic regions (data not shown). The E and M gene phylogenetic analyses showed monophyletic clustering of the strains from North America with some recent strains from China: CH/FJZZ-9/2012 and CH/FJND-3/2011 for E gene and BJ-2011-1, JS-HZ2012, CH/ZMZDY/11, CH/FJZZ-9/2012, and AH2012 for M gene. In contrast, the N gene phylogenetic analysis segregated all of the North American strains from the global PEDV strains, and showed a close phylogenetic relationship between the PEDV strains from North America and some of the recent strains from China: CH/FJZZ-9/2012, CH/FJND-3/2011, BJ-2011-1, AH2012, GD-B, JS-HZ2012, and CH/ZMZDY/11.

Potential Recombinant Origin of the S INDEL Strains

To determine the involvement of recombinant events in evolution of the S INDEL strains from North America, we used RIP to compare strains from North America with the closely related ancestral strains AH2012 and CH/ZMDZY/11 from China and with the historic S INDEL strain CH/S (Figure 3). The highest overall similarity was between non–S INDEL strain USA/Minnesota42/2013 and strain AH2012; similarity was relatively consistent across most of the genome length. The lowest overall similarity was between the North American non–S INDEL strains

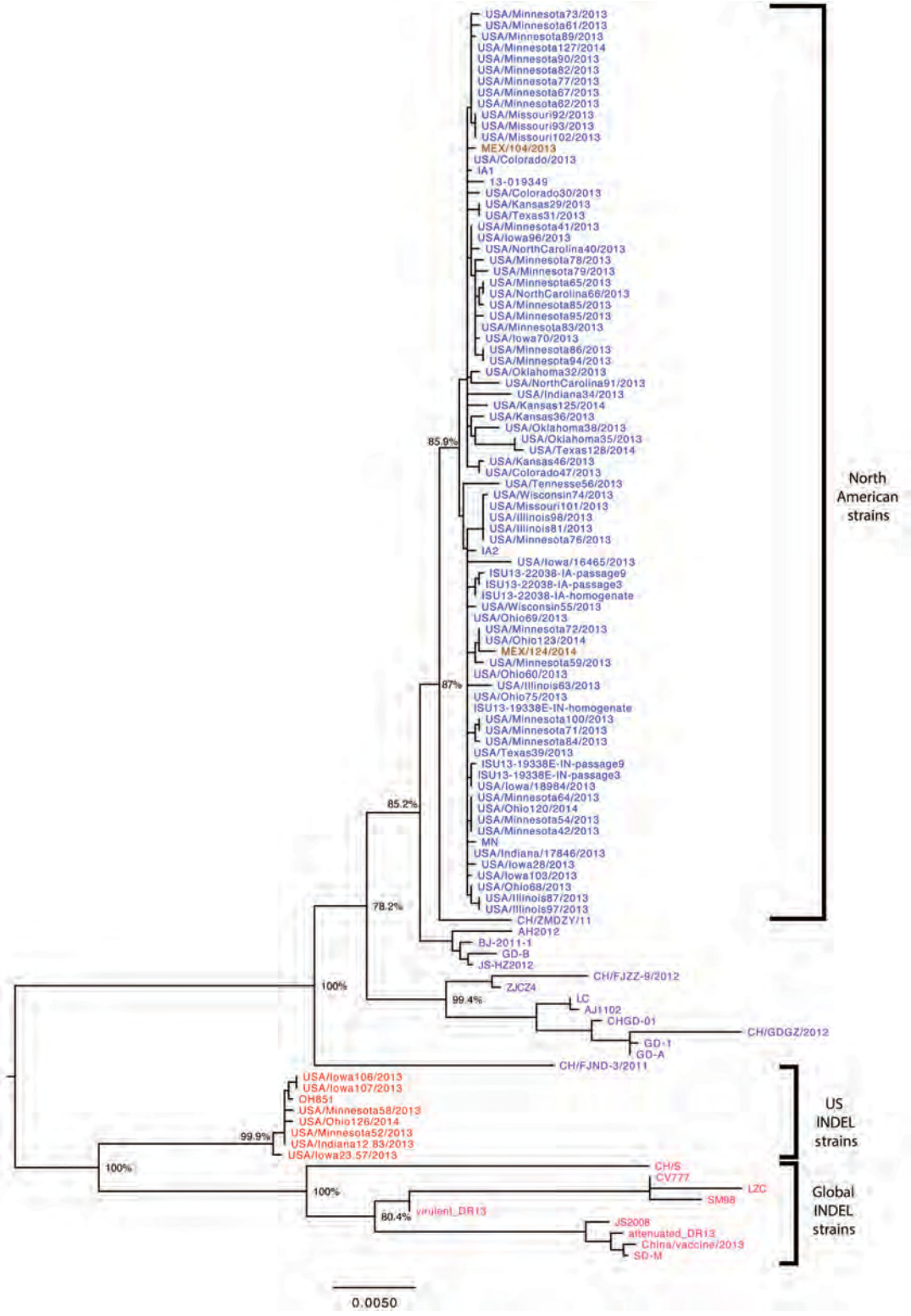

Figure 2. Phylogenetic tree based on the spike gene (S) sequence of 112 North American porcine epidemic diarrhea virus strains. Blue represents US non–S INDEL strains; red represents US S INDEL strains; brown represents Mexican strains; purple represents worldwide non–S INDEL strains; and pink represents global S INDEL strains. Bootstrap values are represented at key nodes. Scale bar indicates nucleotide substitutions per site. CH, China; IA, Iowa; S INDEL, insertions and deletions in the spike gene; IN, Indiana; ISU, Iowa State University; MEX, Mexico; MN, Minnesota; USA, United States of America.

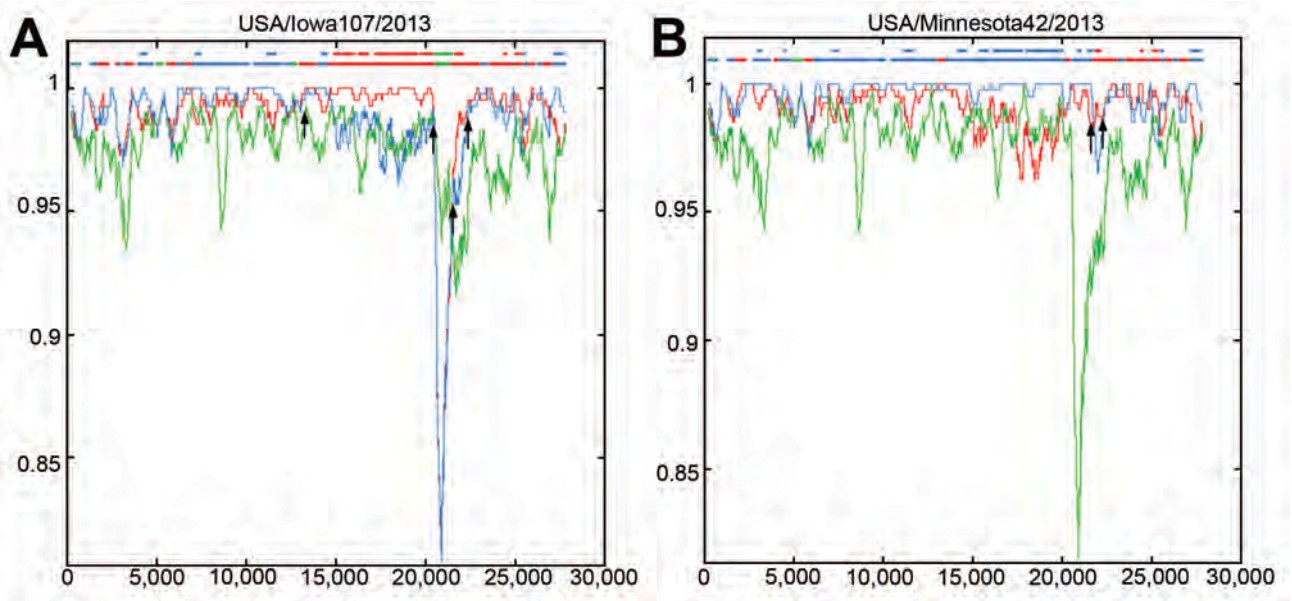

Figure 3. Identification of US porcine epidemic diarrhea virus (PEDV) strains with insertions and deletions in the spike gene as potential recombinant strains. At each position of the window, the query sequence USA/Iowa107/2013 (A) or USA/Minnesota42/2013 (B) was compared with background sequences for 3 strains from China (CH/ZMZDY/11, CH/s, and AH2012). The *x*-axis represents the length of the PEDV genome, and the *y*-axis represents the similarity value. The red line represents PEDV strain CH/ZMDZY/11, the green line represents CH/S, and the blue line represents AH2012. When the query sequence is similar to the background sequence(s), the homologous regions are indicated as thick dashed lines (of the corresponding color) on the top of the plot. Arrows represent potential recombination breakpoints.

and CH/S strain. However, sequence similarity in the S gene (excluding the hypervariable S1 region that was identical to that in AH2012) was higher between the non–S INDEL strains from the North America and CH/ZMZDY/11; this finding is consistent with the phylogenetic analysis results and indicates the presence of potential recombination breakpoints within the S gene. In contrast, the S INDEL strain USA/Iowa107/2013 appeared to possess a composite genome structure: the ORF 1a region was most similar to that of strain AH2012, the ORF 1b region was substantially more similar to that of strain CH/ZMDZY/11, the S gene S1 region was most similar to that of strain CH/S, and the S2 region was most similar to that of strain CH/ZMZDY/11. Therefore, the potential recombination breakpoints in the S INDEL strain genome may have been located between ORF 1a and ORF 1b, between S1 and S2, and between ORF 3 and E gene. The 3′ end (E, M, and, N genes) of the S INDEL and non–S INDEL strains from North America was relatively dissimilar to that of the possible parental strains (AH2012 and CH/ZMZDY/11) that were used as background sequences.

Discussion

Despite the very recent emergence of PEDV in North America, our findings show substantial genetic heterogeneity among the strains. Our findings also confirm, as postulated by others (*25*,*26*), that strain AH2012 from China shares an ancestral strain with the non–S INDEL strains in North America. In addition, we observed an increased representation of the clade I strains at the end of 2013, which may reflect the evolution of PEDV in North America.

These findings raise several questions. First, were there multiple PEDV introductions into North America? Second, were the PEDV S INDEL variants introduced at the same time (April 2013) and are they continuing to cocirculate? Last, do the observed diversity and grouping of PEDV strains from North America represent PEDV evolution within North America, in which S INDEL strains are natural mutants of the originally detected PEDV strains in the United States? The recently identified, dating back to June 5, 2013, S INDEL PEDV strains from the United States possess several distinct molecular traits. These traits create uncertainty regarding the parental strain(s) of these PEDVs and indicate that they could have originated from multiple recombination events before their introduction into the United States. However, none of the potential parental PEDV strains for the origin of the outbreaks in North America has been identified worldwide. Overall, the findings from our phylogenetic and recombination identification analyses did not support the hypothesis of a new PEDV introduction into the United States after April, 2013; instead, our findings highlighted the possibility of multiple parental PEDV strains introduced into North America at the same or similar time.

Identical INDELs and SNPs in the highly immunogenic S1 region of the S gene of the global and the US S INDEL PEDV strains may have been generated through a long chain of recombination events or similar evolutionary mechanisms that enabled PEDV to evade the host immune response. Similar evolutionary mechanisms could have resulted in de novo generation of these specific INDELs in each country-specific PEDV pool. In addition, these S INDELs and the associated SNPs in the hypervariable S1 region of the S gene may represent a stable molecular pattern possibly associated with a less virulent phenotype. The reportedly less virulent variant US PEDV strain, OH851, contained these S INDELs and the SNPs in the ORF 1b region (*27*). These S INDELs are reportedly associated with decreased virulence and are relatively infrequent (in ≈9.5%–14% of PEDV strains), suggesting that they may also be associated with decreased transmission rates, compared with rates for typical highly virulent PEDV strains. The immunologic cross-protection in pigs between S INDEL and non–S INDEL PEDV strains in the United States is unknown, but in Asia, the historic S INDEL vaccines (CV777 lineage) failed to protect against the virulent PEDV strains that emerged after 2010 (*5*). Therefore, whether S INDEL PEDV generation represents a mechanism of adaptation to a partially immune pig population after initial infection with highly virulent non–S INDEL strains is unclear. Most historic (1978–2008) PEDV strains from Europe and Asia contain these INDELs/SNPs in their S protein, suggesting that these changes could contribute to their long-time circulation (endemic) in swine populations, potentially causing only mild disease.

The S INDELs and specific SNPs in the PEDV strains from North America appeared in strong association with a distinct pattern of specific SNPs in the ORF 1b region, which shares a common recent ancestor with strain CH/ZMZDY/11, but the S1 region of the S gene could have been provided by another ancestral strain. A comparison of the complete genome sequences of strain DR13, a virulent PEDV strain from Korea, and its attenuated counterpart showed specific changes in ORFs 1ab and 3 and in S and E genes of the attenuated strain; as a result of these changes, the ORFs and genes in the attenuated strain are shorter than those in the virulent strain (*20*). However, in contrast to the findings for the virulent–attenuated DR13 strain pair and the strong evidence of the role of ORF 3 alterations in the pathogenesis of or cell culture adaptation of feline infectious peritonitis virus (*31*), we did not observe any of the following: characteristic changes in the ORF 3–E gene region, other than S INDELs; a potentially recombinant ORF 1b region; or any reports of decreased severity of PEDV in the field. The higher level of nucleotide conservation of the North American PEDV strains in the E and M genes and greater genetic variability of N gene may be representative of geographic specificity rather than virulence. The mechanisms governing the virulence of PEDV (or coronaviruses in general) may be redundant, or the S INDEL strains may be an intermediate between those of virulent and attenuated PEDVs (e.g., lower virulence).

Our findings provide evidence of the complex and rapid evolution of PEDV strains in North American swine herds. In addition, our findings suggest that the S INDEL strains, which are likely of recombinant origin, may represent complementary PEDV mechanisms that enable the spread and persistence of the virus in swine populations after exposure to highly virulent PEDV strains. This possibility should be confirmed by thoroughly assessing the immune responses to PEDV in the US swine that were sources of the S INDEL PEDV variant strains and by conducting experimental pig inoculation and cross-protection studies. Active epidemiologic surveillance and immunologic studies are urgently needed to understand PEDV evolution worldwide and to develop optimal PEDV vaccines.

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Person-to-Person Household and Nosocomial Transmission of Andes Hantavirus, Southern Chile, 2011

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Andes hantavirus (ANDV) causes hantavirus cardiopulmonary syndrome in Chile and is the only hantavirus for which person-to-person transmission has been proven. We describe an outbreak of 5 human cases of ANDV infection in which symptoms developed in 2 household contacts and 2 health care workers after exposure to the index case-patient. Results of an epidemiologic investigation and sequence analysis of the virus isolates support person-to-person transmission of ANDV for the 4 secondary case-patients, including nosocomial transmission for the 2 health care workers. Health care personnel who have direct contact with ANDV case-patients or their body fluids should take precautions to prevent transmission of the virus. In addition, because the incubation period of ANDV after environmental exposure is longer than that for person-to-person exposure, all persons exposed to a confirmed ANDV case-patient or with possible environmental exposure to the virus should be monitored for 42 days for clinical symptoms.

Hantavirus cardiopulmonary syndrome (HCPS) is caused by infection with New World hantaviruses. First described in 1993 in the southwestern United States, HCPS has been documented throughout the Americas (*1*,*2*). For human cases, the mean incubation period of hantavirus infection from exposure to illness onset is 18.5 (range 7–42) days (*3*). As of December 31, 2013, a total of 848 human HCPS cases had been reported in Chile; the case-fatality rate has ranged from 32% to 35% per year (*4*).

The sole confirmed etiologic agent of HCPS in Chile is Andes virus (ANDV). Human infection with this virus occurs from exposure to contaminated excreta and secretions of rodents of the family *Cricetidae*. Transmission of ANDV between rodents has been experimentally documented after exposure of seronegative rodents to inhalation of aerosolized infected rodent secretions (*5*). ANDV is endemic in Chile and Argentina and is the only hantavirus for which person-to-person transmission has been documented. Person-to-person transmission of ANDV occurs mainly in family clusters or, less commonly, after activities in which close contact with an infected case-patient has occurred, primarily during the disease prodrome (*6*–*8*). A prospective study in Chile found that sexual partners and other close household contacts of ANDV-infected persons showed a 10-fold higher risk of acquiring the virus than household contacts who did not share bed or bedroom with the index case-patient (*3*,*9*).

Nosocomial transmission of ANDV has been a matter of concern for infection control practice and for health care workers who provide care for these patients, and in particular for workers who perform invasive procedures. In Argentina, person-to-person transmission of ANDV was documented in a physician who acquired infection after exposure to an ANDV-infected patient shortly after onset of the febrile prodrome (*7*,*8*). Although person-to-person transmission in Chile has been epidemiologically documented (*10*), nosocomial transmission has not been reported. Seroprevalence studies conducted among health care workers in hospitals in Chile where patients with ANDV infection have been treated have reported that health care workers exhibited ANDV IgG antibody at a proportion similar to that of the general population (*11*,*12*). Similarly, a study in the southwestern United States found no evidence of nosocomial transmission of another hantavirus, Sin Nombre virus (*13*).

We describe an outbreak of 5 cases of ANDV infection that occurred in a small, rural community in southern Chile in 2011. We present the epidemiologic and the clinical features of the cases, along with the molecular analysis of the

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virus strains from each case. Epidemiologic and virus sequence analyses support person-to-person transmission of ANDV in 4 of these cases, including nosocomial transmission in 2 cases.

Materials and Methods

Study Population

A case cluster of 5 human case-patients, including 2 persons involved in health care, occurred in Corral, Los Rios, Chile, during February–April 2011. Clinical history and information from epidemiologic questionnaires were obtained for each patient; all 5 had an acute febrile illness and signs and symptoms compatible with hantavirus infection. Acute infection was confirmed by detection of IgM against viral nucleoprotein antigen and real-time reverse transcription PCR (RT-PCR) targeting the small RNA segment of ANDV in blood samples obtained from these patients during the acute illness (*14*,*15*). Samples from 7 additional patients who had had HCPS in the same geographic region in previous years were used as controls for virus sequence analysis. All participants signed an informed consent approved by an ethics committee.

Geographic and Demographic Features of Corral

Corral is a coastal town (39°52′0″ S, 73°25′60″ W) located 15 km west of Valdivia, the capital of the Los Rios region in Chile; the town is in the foothills of a coastal mountain range in the Valdivian rainforest ecoregion (*16*). The population is ≈5,433 inhabitants. Corral has 1 primary care hospital with 5 beds (hospital I); all patients with complications are transferred to a regional care center in Valdivia that has intensive care facilities (hospital II). Since 1997, a total of 13 cases of hantavirus infection have been reported in Corral, including the 5 cases described in this report (*17*). Prior to this report, the last 2 confirmed cases were in 2008 and 2010.

Outbreak Description

On March 20, 2011, two suspected cases of hantavirus infection were reported. The patients were a 31-year-old woman (case-patient B) who worked as a nursing assistant at hospital I and a 53-year-old woman (case-patient C). Both lived near Corral. In addition to these cases, in late February, a 73-year-old man (case-patient A), the spouse of case-patient C, had been transferred from hospital I to hospital II for treatment of a pulmonary disease and evaluated for hantavirus infection; initial serologic testing results at a national reference laboratory were negative. On March 22, a fourth patient (case-patient D), a 60-year-old female housekeeper at hospital I, was admitted to hospital II with respiratory failure; she died a few hours later. A fifth patient (case-patient E), a 34-year-old man who was the husband of case-patient B, was hospitalized on April 3 at hospital II. On April 3, an epidemiologic investigation was initiated by the Health and Epidemiology Service, including investigation of infection control measures used at hospital I.

Genetic Characterization of the Virus

RNA was obtained from patients' leukocytes from diagnostic samples and extracted by using the High Pure Viral RNA Kit (Roche Diagnostic GmbH, Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions. For segment amplification, heminested RT-PCR was performed as described previously (*16*). Two portions of the medium segment, Gn and Gc, were amplified (Table 1), and the amplicons underwent agarose gel purification and sequencing in both directions. The chromatogram of each sequence was analyzed and aligned to generate a consensus sequence by using BioEdit version 7.1.11 (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Twelve sequences were aligned by using ClustalW (http://www.clustal.org). Sequences were phylogenetically analyzed by conducting maximum-likelihood (ML) and Bayesian methodology on the concatenated Gn and Gc sequences. For ML, PAUP* version 4.0 (*18*) was used for a heuristic search with 100 random additions and branch swapping via tree-bisection-reconnection (*19*). jModeltest 3.7 was used to choose the best-fitting model of sequence evolution (*20*). The corrected Akaike information criterion (Akaike 1974) identified the Kimura 81 unequal base frequencies + gamma model (K81uf + Γ) as optimal (–lnL = 1251.2770, Akaike information criterion = 2515.7539, G = 1.5780), with base frequencies A = 0.2868, C = 0.3132, G = 0.0670, and T = 0.3329. Reliability of nodes in the ML tree was estimated by bootstrap analysis (*21*) obtained after 1,000 pseudo-replicates. The tree was rooted on the basis of the outgroup criterion by using the ANDV sequence available in GenBank (accession no. NC_003467.2). Sequences also were analyzed in a Bayesian framework to estimate the posterior probabilities of phylogenetic trees. Ten million phylogenetic trees were generated; the first 1,000 trees of the sample were removed to avoid including trees before convergence of the Markov Chain. As 2 independent molecular markers were used, a general likelihood–based mixture model of sequence evolution was applied as described (*22*). This model accommodates cases in which different sites in the alignment evolved in qualitatively distinct ways but does not require prior knowledge of these patterns or

Table 1. Primers used for M segment amplification and sequencing of Andes hantavirus

Primer identification	Primer sequences, 5′ → 3′
GN1+	TAGTAGTAGACTCCGCAAGAAGAAG
GN534–	TCCTGCTKKTAAACACACTAGCCAT
GC94+	TGCAAATGATTGTGTTAGTAACACCA
GC674–	GTATTAGAGCCCCTAGCACAGGTT

partitioning data. These analyses were conducted by using Bayes Phylogenies software (*22*). To find the best mixture model of evolution, the number of general time reversible matrices was estimated by using a reversible-jump Markov chain Monte Carlo method (*23*).

Results

Laboratory and Epidemiologic Investigations

IgM and IgG against ANDV were detected in serum samples, and ANDV RNA was detected by RT-PCR in blood for all 5 patients in the cluster (Table 2; Figure 1). Case-patient A, the 73-year-old man, was identified as the index case-patient of the cluster.

Case-patient A lived in a small settlement near Corral. His main risk activity was the cleaning of a home cellar where he was moving tiles on February 5. The cellar was heavily contaminated with rodent feces. The patient was admitted to hospital I on February 24 after 3 days of fever, dry cough, weakness, and progressive dyspnea. During hospitalization, he experienced progressive respiratory compromise, productive cough, and intense sweating that required frequent changes of gowns, sheets, and blankets. On February 26, he was transferred to the critical care unit at hospital II for mechanical ventilation. Serum samples were sent to the National Reference Laboratory 11 days after onset of his symptoms; results were negative for ANDV IgM. When the epidemiologically related hantavirus case-patients were admitted to hospital II, ANDV IgM testing was repeated, 24 days after onset of his symptoms, and results were positive. Case-patient A died on March 26 after 28 days of mechanical ventilation and use of vasoactive drugs.

Case-patient B, a nursing assistant at hospital I, exhibited a fever on March 17. She was hospitalized on March 20 and the same day was transferred from hospital I to the intensive care unit at hospital II. Severe shock and respiratory failure developed, and high doses of vasopressors and mechanical ventilation were required. A diagnosis of HCPS caused by ANDV infection was confirmed after 8 days of symptoms, and she was discharged on April 11. This patient had direct contact with case-patient A at hospital I from February 24–26, during his febrile prodrome and progression to the cardiopulmonary phase. She changed the patient's clothes, sheets, and blankets because he perspired profusely. In addition, having met the patient previously, she greeted case-patient A with a kiss on his cheek several times during his hospitalization.

Table 2. Clinical and epidemiologic features of 5 patients involved in outbreak of ANDV infection, Chile, 2011*

Feature	Case-patient A†	Case-patient B	Case-patient C	Case-patient D	Case-patient E
Age, y/sex	73/M	31/F	53/F	60/F	34/M
Occupation	Farmer	Nursing assistant at hospital	Teacher	Cleaning personnel at hospital	Car mechanic
Relationship to other case-patients	Husband of case-patient C	Health care provider for case-patient A	Wife of case-patient A	Health care assistant for case-patient A	Husband of case-patient B
Date of symptom onset	Feb 21	Mar 17	Mar 18	Mar 18	Apr 2
Date of hospitalization	Feb 24	Mar 20	Mar 20	Mar 22	Apr 3
Signs and symptoms					
Fever	Yes	Yes	Yes	Yes	Yes
Respiratory symptoms‡	Yes	Yes	No	Yes	Yes
Gastrointestinal symptoms§	No	No	Yes	Yes	No
Other symptoms¶	No	Yes	Yes	Yes	Yes
Mechanical ventilation, d	28	8	0	1	6
Hospitalization, d	30	22	12	1	17
Outcome	Died	Survived	Survived	Died	Survived
Days from environmental exposure to onset of symptoms	16	25–26	41	7–45	41–42
Days from exposure to hantavirus case-patient to onset of symptoms	NA	19–21	22–25	18–20	13–27
Laboratory test results on admission					
Platelet count, × 10^3/μL	51	56	108	101	147
Leukocytes, × 10^3 cells/μL	4,67	5,46	1,21	3,92	11,46
Hematocrit, %	52	39	39	45	44
Lymphocytes, %	12	7	39	19	7
Immunoblasts, %	Yes	Yes	Yes	NR	Yes
IgM/IgG for ANDV	Negative#	Positive	Positive	Positive	Positive
RT-PCR ANDV in blood cells	ND	Positive	Positive	Positive	Positive

*ANDV, Andes virus; NA, not applicable; NR, not reported; RT-PCR, reverse transcription PCR; ND, not done.
†Index case-patient.
‡Dry cough, dyspnea, cyanosis, crepitus.
§Vomiting, diarrhea, nausea.
¶Severe headache, meningeal signs, myalgia, arthralgia, conjunctival infection, chills, photophobia, facial edema.
#On hospital admission. Repeat testing after 24 days yielded positive results.

She also had close contact with her husband at their home from the time she cared for the index case-patient through the first 3 days of her illness. She recalled possible environmental exposure from camping at 2 local beaches during February 1–4 and February 19–20; she collected wood and cleaned the area to set up tents.

Case-patient C was the spouse of case-patient A. She shared the same bed and cared for him during his febrile prodrome but denied that they had sexual activity after symptom onset. She entered the contaminated cellar with her husband but did not participate actively in his work in this area. On March 18, twenty-five days after her husband's illness onset and 41 days after they entered the cellar, she exhibited mild fever, severe headache, myalgia, and photophobia . She sought medical attention at hospital II while her husband was still hospitalized, and acute ANDV infection was confirmed on March 24. Her chest radiograph results were normal. Her most remarkable symptoms were headache and irritability, and she had meningeal signs. Testing of cerebrospinal fluid (CSF) showed 8 white mononuclear cells, normal glucose levels, and a slightly elevated protein level of 0.5 g/L. CSF testing by RT-PCR for ANDV and ELISA for ANDV-specific IgG yielded negative results.

Case-patient D, a housekeeper at hospital I, had fever, abdominal pain, and vomiting develop on March 18. Two days later, she was hospitalized at hospital I, and 4 days later, she was transferred to hospital II, where severe shock and respiratory failure developed. She died a few hours after admission to hospital II. Her diagnosis was confirmed by positive results of serologic testing and RT-PCR for ANDV. She had direct and indirect contact with case-patient A while he was at hospital I. She entered his room and helped the nursing assistant (case-patient B) change his clothes and remove his sheets and bedclothes for washing.

Case-patient E, the husband of case-patient B, had fever, headache, myalgia, and back pain develop on April 2, and he was admitted to hospital II on April 3. Serologic testing for ANDV IgM and IgG after 5 days of symptoms yielded negative results, but results of RT-PCR for ANDV RNA were positive. IgM and IgG seroconversion were confirmed 10 days after symptom onset. The person-to-person exposure period for this patient was March 6–20; his possible environmental exposure exceeded the known incubation period for ANDV (*11*). Shock and respiratory failure developed, and he required mechanical ventilation and vasopressors but survived.

Environmental Investigation

Rodent trapping was performed for 2 and 3 nights, respectively, at the 2 sites where case-patients reported possible environmental exposure: the cellar of the home of

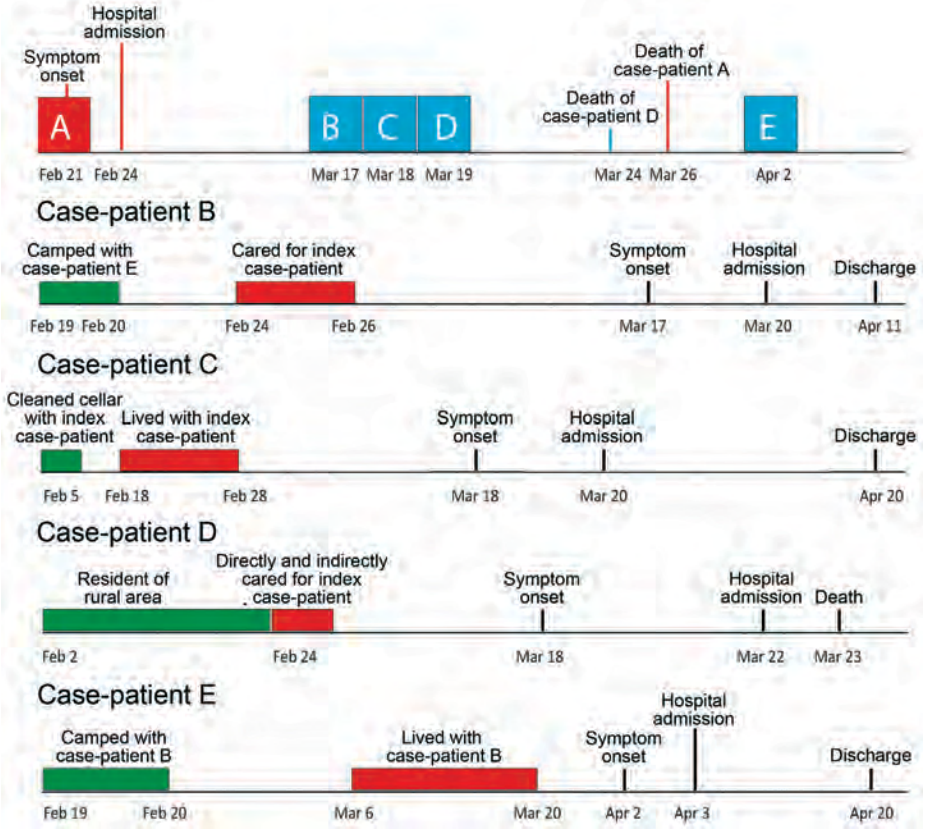

Figure 1. Timelines showing progression and key events related to each case-patient (A–E) in a cluster of 5 Andes hantavirus cases, southern Chile, 2011. Blue boxes along timeline for index case-patient (A) indicate date of illness onset for subsequent case-patients; green boxes indicate environmental exposures (exposure for case-patient A was the same as for case-patient C); red boxes indicate contact with other case-patients.

case-patients A and C and a camping area used by case-patients B and E (Table 3). Rodent serum samples were tested for ANDV antibodies by strip immunoblot assay (*24*); results were positive for 1 *Abrothrix longipilis* rodent trapped at the camping site. However, RT-PCR results for this sample were negative, and testing of rodents trapped at the home of case-patients A and C yielded negative results.

Viral Molecular Analysis

A portion of 942 bp of the ANDV small RNA segment was amplified and sequenced from samples of each of the 5 patients in the case cluster. Sequences aligned by using ClustalW showed 100% identity (data not shown), an observation consistent with the high degree of conservation of the small segment among hantaviruses (*7*,*25*).

Virus variability was established by comparing a portion of 914 bp of the highly variable ANDV medium RNA segment. The sequences obtained for the 2 medium segments encoding the ANDV glycoproteins Gn and Gc were compared separately (data not shown) and concatenated. Results were visualized in the identity matrix of concatenated sequences and showed that the concatenated sequences derived from the 5 cases in the cluster were similar to each other but differed from viral sequences from 7 patients who acquired ANDV in the same community in previous years (Table 4). The molecular identity of the concatenated Gn and Gc sequences between cases ranged from 99% to 100%, whereas the comparison with control sequences from the same geographic region ranged from 97% to 99%. These values show higher identity between the sequences derived from the cluster cases compared with other human cases from the same geographic region from previous years. All sequences obtained in this study have been deposited in GenBank (accession nos. KC567258–KC567281).

The phylogenetic analyses through ML and Bayesian methods revealed similar topologic results; thus, a single tree is shown (Figure 2). Results show 2 major groups with strong support provided by the bootstrap and posterior probability values. The group of samples that included the Corral cases is clearly separated from other major clustering that includes ANDV sequences from other localities in Chile.

Discussion

ANDV is the only hantavirus for which person-to-person transmission has been reported (*7*). Our study of a case cluster in Chile provides epidemiologic and molecular evidence that strongly supports the conclusion that 4 of 5 cases resulted from person-to-person transmission of ANDV, including 2 cases of nosocomial transmission.

Most of the reports of person-to-person transmission of ANDV share common traits that constitute potential risk factors for virus spread (*7*–*9*). These features were also observed in this cluster. First, the period of the disease during which the acute case-patient and the household contact or health care personnel have close contact is primarily the febrile prodrome phase, when symptoms are nonspecific for hantavirus. Second, the number of days from exposure to an index case-patient and the onset of symptoms among additional cases ranges from 12 to 27 days (*7*,*26*), consistent with the intervals observed in our report. In the 2 cases for which environmental exposure was reported, the estimated incubation period after that exposure exceeded the longest reported incubation range of 42 days for ANDV (*3*,*11*). In contrast, in these 2 cases the estimated incubation periods from exposure to a case-patient to onset of symptoms was 13–27 days. Finally, the viral genetic characterization established that viruses from the case cluster shared a high nucleotide sequence identity in Gn and Gc fragments, the most variable viral genomic regions (*6*).

During the prodrome, when symptoms are nonspecific, consideration of ANDV infection and early diagnosis might be triggered by a history of environmental exposure (*1*,*2*) or close exposure to another confirmed case-patient within the known incubation period (*3*,*6*). In this cluster, all the cases appeared in a geographic region that is considered an endemic risk area for hantavirus (*26*,*27*). However, no other cases had occurred in this town since 2010, and our epidemiologic and virus sequence analysis showed that the main risk factor for all the 4 additional cases was the patient's close contact with a symptomatic, HCPS case-patient (*6*,*28*).

One case of nosocomial transmission of the virus has been previously reported in Argentina (*7*), and evidence of this transmission has been sought in Chile (*12*). We document 2 cases of nosocomial transmission of ANDV, from the index case-patient to a nursing assistant and to a housekeeper, even though their contact with the patient was limited to kissing the patient on the cheek and to

Table 3. Results of environmental investigation for 4 cases of ANDV infection, Chile, 2011*

Case-patients	Days after case-patient diagnosis	No. trapping nights	No. trapped rodents	No. traps per night	Rodent species trapped	SIA results, n = 24	RT-PCR results
B and E	63	3	46	57, 40, 40	*Abrothrix longipilis, A. olivaceus*, other *Abrothrix* sp., *Oligoryzomys longicaudauts*	1 positive (*A. longipilis*)	Negative
A and C	90	2	9	68, 68	*A. olivaceus, O. longicaudauts, Rattus norvegicus, R. rattus*	Negative	ND

*SIA, strip immune assay; RT-PCR, reverse transcription PCR; ND, not done.

Table 4. Identity matrix of concatenated Gn and Gc sequences of ANDV isolates from the 5 case-patients in this study compared with sequences from ANDV samples from previous case-patients in the same geographic region of Chile*

Sequence	Pan2010	Pai2011	Mar2010	Fut2010	C2012(1)	C2012(2)	Pan2012	C	B	E	D	A
Pan2010	–	0.972	0.991	0.985	0.964	0.971	0.989	0.961	0.961	0.955	0.961	0.961
Pai2011		–	0.974	0.973	0.983	0.994	0.976	0.984	0.984	0.978	0.984	0.984
Mar2010			–	0.987	0.970	0.975	0.995	0.961	0.961	0.955	0.961	0.961
Fut2010				–	0.964	0.974	0.990	0.960	0.960	0.953	0.960	0.960
C2012(1)					–	0.985	0.970	0.970	0.970	0.963	0.970	0.970
C2012(2)						–	0.978	0.981	0.981	0.974	0.981	0.981
Pan2012							–	0.963	0.963	0.957	0.963	0.963
C								–	1.000	0.993	1.000	1.000
B									–	0.993	1.000	1.000
E										–	0.993	0.993
D											–	1.000
A												–

*Geographic location and year are indicated for control cases (numbers in parentheses indicate multiple cases in the same year; case-patient identification letters (A–E) are given for cases from this study. – indicates alignment of the same sequence.

handling bedding and gowns (no invasive procedures). Two seroprevalence studies performed soon after recognition of hantavirus in Chile did not reveal a higher proportion of antibodies against ANDV among hospital personnel when compared with the general population (*11*,*12*).

In our study, ANDV infection was not diagnosed in the index case-patient until he had been ill for 31 days, which resulted in a wider time frame of exposure for health care personnel. The patient had a history of diabetes mellitus but no history of any other immunodeficiency that might explain his initial negative serologic test. However, the initial testing was not repeated, so we cannot rule out the possibility of a false-negative result.

For case-patient C, the clinical manifestation of illness was unusual because she lacked respiratory symptoms and showed meningeal irritability as the main sign of the infection. Viral RNA and specific antibodies were not detected, but a slight elevation in the CSF white blood count and protein level were seen. It is possible that viral RNA was present before CSF testing or that it was below the level of detection by RT-PCR, but the timing of her symptoms is probably inconsistent with a postinfectious process.

It is not clear why person-to-person transmission has been documented for ANDV but not for other hantaviruses. Risk factors associated with close contact, including sexual contact, deep kissing, or sleeping in the same bed or room, have been identified in a prospective study of household contacts of index case-patients with HCPS (*9*). As such, respiratory secretions, saliva, or both may be involved in transmission. Puumala virus RNA has been detected by RT-PCR but not by cell culture in saliva from patients who had hemorrhagic fever with renal syndrome (*29*). The antiviral activity of different human saliva concentrations has been experimentally tested against Hantaan virus, Puumala virus, and ANDV; ANDV was least sensitive to the antiviral effect of saliva (*30*). RT-PCR testing has found ANDV RNA in previous and ongoing studies in blood and in body fluids, including gingival crevicular fluid, saliva, endotracheal fluid, and urine (*31*). ANDV was isolated from blood obtained from a child in Chile before the onset of symptoms or development of ANDV antibodies (*32*), and studies are ongoing to determine which, if any, of the body fluids positive by RT-PCR also contain infectious virus.

To characterize and compare the outbreak viral sequences, we used as reference material a selection of sequences from strains obtained 2 or 3 years earlier in the same ecogeographic region near Corral. All 5 medium fragments obtained from case-patient isolates in this cluster were highly similar to each other but were more distantly related to the reference sequences. The strong relatedness of the viruses in the Corral cluster is supported by high bootstrap and posterior probability values in the phylogenetic analyses. Furthermore, the small segment showed 100% identity between the 5 sequences in this cluster. The dates of exposure to high-risk environments or to persons with ANDV infection, known incubation periods, and 100% sequence identify all support a conclusion of person-to-person transmission (*7*). Our data showed 99%–100% identity for a fragment of 913 bp of the medium segment, supporting identity using different sequences. However, we did not include noncoding region fragments, which might provide additional confirmation of identity.

Our study documents a small but definite risk of nosocomial acquisition of ANDV infection for personnel who care for patients, including handling of bedding and gowns. After this investigation, the Ministry of Health of Chile has recommended, in addition to strict adherence to universal precautions, the use of droplet precautions when ANDV infection is suspected. Use of N95 respirator masks, designed to prevent the inhalation of airborne particles, is recommended for those procedures associated with aerosolization of viral contaminated secretions (e.g., respiratory, saliva) when procedures such as suction or intubation are performed. However, this recommendation should be extended to all personnel who have any kind of direct contact with patients or body fluids, including bedding and gowns.

Finally, all close household contacts and health care personnel exposed to a confirmed ANDV case-patient should be closely monitored for signs and symptoms of

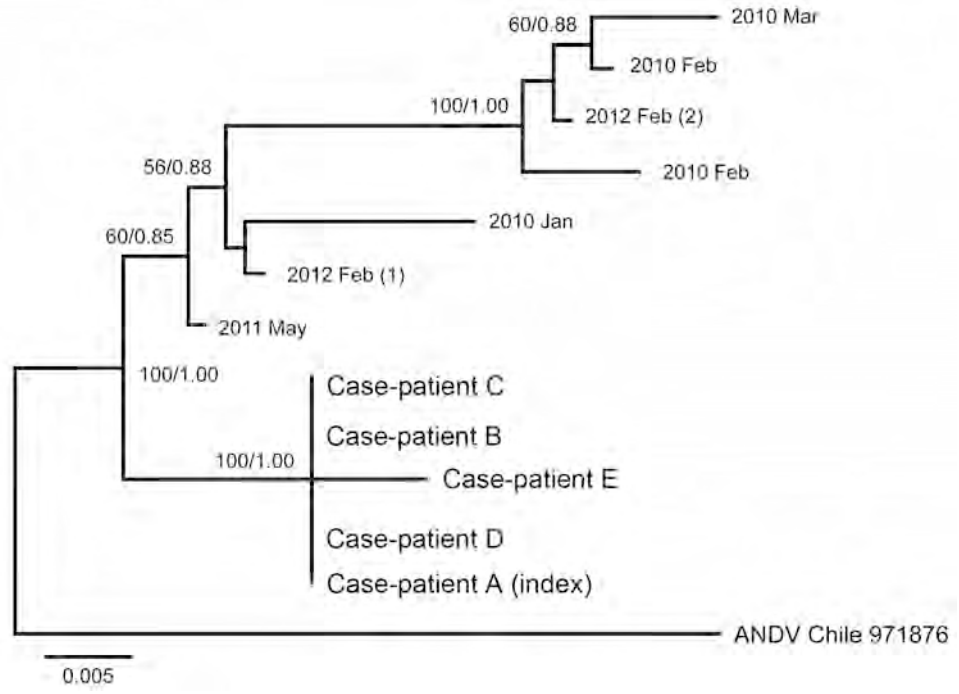

Figure 2. Phylogenetic analyses of the medium RNA segment (Gc and Gn) of concatenated sequences of Andes hantavirus (ANDV). Isolates from the case-patients (A–E) from the 2011 outbreak in Chile were compared with control samples from the same geographic region (indicated by year isolated; number in parentheses indicates multiple isolates from the same year) and an ANDV sequence from GenBank (bottom isolate on tree; accession no. NC_003467.2). Scale bar indicates substitutions per site.

infection, such as fever, myalgia, headache, and abdominal pain, during the entire documented incubation period of 42 days, even though in person-to-person transmission of ANDV, the onset of symptoms has usually occurred 12–27 days after close contact with a sick patient (*6*,*9*). ANDV RT-PCR should be performed in addition to testing for specific IgM in any exposed contact in whom fever develops within the incubation period, particularly if testing is done within a few days of the onset of fever and before onset of the cardiopulmonary phase. Results of ANDV RT-PCR on blood cells may be positive as early as 5–15 days before onset of symptoms or detection of ANDV antibody (*9*). As we have documented, RT-PCR can detect ANDV RNA in the rare, symptomatic patient in whom seroconversion is delayed.

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Effects of Mefloquine Use on *Plasmodium vivax* Multidrug Resistance

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Numerous studies have indicated a strong association between amplification of the *multidrug resistance-1* gene and in vivo and in vitro mefloquine resistance of *Plasmodium falciparum*. Although falciparum infection usually is not treated with mefloquine, incorrect diagnosis, high frequency of undetected mixed infections, or relapses of *P. vivax* infection triggered by *P. falciparum* infections expose non–*P. falciparum* parasites to mefloquine. To assess the consequences of such unintentional treatments on *P. vivax*, we studied variations in number of *Pvmdr-1* (PlasmoDB accession no. PVX_080100, NCBI reference sequence NC_009915.1) copies worldwide in 607 samples collected in areas with different histories of mefloquine use from residents and from travelers returning to France. Number of *Pvmdr-1* copies correlated with drug use history. Treatment against *P. falciparum* exerts substantial collateral pressure against sympatric *P. vivax*, jeopardizing future use of mefloquine against *P. vivax*. A drug policy is needed that takes into consideration all co-endemic species of malaria parasites.

Since World War II, antimalarial drugs have been intensively used to prevent or treat malaria (*1*). As observed with other antimicrobial agents, their use (or frequent misuse, when malaria diagnosis was based only on clinical symptoms without parasitologic confirmation) led to the emergence, selection, and spread of resistant parasites (*2*). This resistance became a global problem during the 1960s, when *Plasmodium falciparum* parasites developed resistance to chloroquine, the most widely used antimalarial drug at that time (*3*). In particular, resistant parasites that emerged in the Greater Mekong subregion of Asia later spread to Africa, triggering a dramatic increase in malaria and malaria-related deaths, particularly among children (*4*). During the 1980s, a similar scenario was observed with sulfadoxine-pyrimethamine (SP) when this association was recommended to replace chloroquine as first-line treatment in uncomplicated falciparum malaria (*5*,*6*). Since then, biological and molecular investigations using laboratory and field isolates have demonstrated that resistance of *P. falciparum* to antimalarial drugs is mediated by 2 major mechanisms: 1) a modification of the parasite target (i.e., mutations in *dihydrofolate reductase* [*dhfr*] or in *dihydropteroate synthetase* [*dhps*] genes) or 2) an increase of the efflux of the drug away from its site of action (i.e., mutations in the *chloroquine resistant transporter* gene or the *multidrug resistance-1* [*mdr-1*] gene or in an increased number of copies of the *mdr-1* gene). These molecular events have been intensively studied and are well known for *P. falciparum* but not for other *Plasmodium* species, mainly because of the ability to culture in vitro *P. falciparum* erythrocytic stages.

Our understanding of the molecular mechanisms of antimalarial drug resistance developed by *P. vivax* is less comprehensive. Although *Aotus* and *Saimiri* monkey models have provided useful information about *P. vivax* biology, most of the data have been gained through comparative studies investigating polymorphisms in orthologous genes encoding resistance to pyrimethamine (*dhfr* gene), sulfadoxine (*dhps* gene), or chloroquine (*chloroquine resistant transporter* or *mdr-1* genes). For instance, mutations in

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codons 57, 58, 61, 117, and 173 of *P. vivax* DHFR (corresponding to codons 51, 59, 108, and 164 of *P. falciparum* DHFR) are involved in resistance to pyrimethamine, although *P. vivax* infections are not usually treated directly with SP (*7*). This resistance was confirmed by heterologous expression studies, invalidating the common idea that *P. vivax* was "intrinsically resistant" to pyrimethamine (*8*), which suggests that the high frequency of mixed *P. falciparum*/*P. vivax* infections that are not detected by microscopy (*9*–*11*) or relapses of *P. vivax* infection after *P. falciparum* infections probably exposes *P. vivax* parasites to antimalarial drugs used to treat falciparum malaria infections, especially those with a long half-life, and selects *P. vivax* genetic traits conferring antimalarial drug resistance.

The impact of antimalarial drugs, especially those with long half-lives (such as mefloquine), on the sympatric *Plasmodium* species is not clearly understood. In areas where *P. falciparum* and *P. vivax* are co-endemic, such as South America and Southeast Asia, mefloquine has been widely used (alone in monotherapy or in combination with artemisinin derivatives) to treat uncomplicated falciparum malaria (*12*). In both areas, emergence of *P. falciparum* parasites resistant to mefloquine has been demonstrated from therapeutic efficacy studies (treatment failure) or in vitro testing (increased IC_{50} [half maximal inhibitory concentration]) and has been associated with the amplification of *P. falciparum mdr-1* (*Pfmdr-1*) gene (*13*–*16*). Recently, several studies performed on *P. vivax* samples collected in Southeast Asia (Thailand, Laos, Cambodia, and Myanmar) (*17*–*19*), South America (Brazil, Honduras) (*20*,*21*), and Africa (Mauritania) (*22*) have shown that *mdr-1* amplification does occur in *P. vivax*.

In this context, and to confirm the impact of the mefloquine drug pressure on *P. vivax* parasite populations, we used a real-time PCR to assess the number of *P. vivax mdr-1* (*Pvmdr-1*) gene copies to evaluate the worldwide distribution of *Pvmdr-1* amplification in samples collected from travelers with vivax malaria returning to France and from residents in areas with different histories of mefloquine use (French Guiana, Cambodia, Madagascar, and Sudan).

Materials and Methods

Sample Collection

P. vivax and *P. falciparum* samples from Madagascar were collected during 2006–2007 as part of the antimalarial drug resistance network, from symptomatic patients before treatment in 19 health centers located in areas of Madagascar with different epidemiologic patterns of malaria transmission: northern (Antsiranana, Antsohihy, Andapa), western (Mahajunga, Miandrivazo, Maevatanana, Morondava, Tsiroanomandidy, Ampasimpotsy), central (Saharevo, Moramanga), southern (Ihosy, Ejeda, Tolagnaro, Iakora, Ranostara, Toliara), and eastern (Farafangana, Toamasina). In Cambodia, *P. vivax* and *P. falciparum* isolates were obtained from symptomatic persons during 2010 in Pailin and Kratie Provinces. Other *P. vivax* samples were collected 1) from malaria-infected travelers returning to France after visiting Africa (Côte d'Ivoire, Ethiopia, Madagascar, and Mauritania), South America (Bolivia, Brazil, Colombia, French Guiana, Venezuela), and Asia (Bangladesh, Cambodia, India, Indonesia, Laos, Malaysia, Nepal, Pakistan, Sri Lanka) during 1997–2009 and were provided by the National Reference Center for Malaria (Paris, France); and 2) from symptomatic *P. vivax*–infected persons in French Guiana (2000–2003) or Sudan (2007).

DNA Extraction and PCR Detection of *P. falciparum* and *P. vivax*

We extracted parasite DNA from blood spots with Instagene Matrix (BioRad, Marnes-la-Coquette, France) or from whole blood samples using the phenol-chloroform method (*23*) or the QIAamp DNA Blood Mini Kit (QIAGEN, Courtaboeuf, France), according to the manufacturer's instructions. Molecular detection and identification of *Plasmodium* parasites were performed by using real-time PCR as described by Chou et al. (*24*).

Determination of the Number of *Pfmdr-1* Copies in Isolates from Cambodia and Malagasy

We measured number of *Pfmdr-1* copies using CFX96 real-time PCR (BioRad) relative to the single copy of the *β-tubulin* (used as a reference gene). Briefly, PCRs were conducted in 25-mL volumes in a 96-well plate containing 1X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia), 0.3 mM of each forward and reverse primer (*Pfmdr-1*, 5′-TGCATCTATAAAACGATCAGACAAA-3′ and 5′-TCGTGTGTTCCATGTGACTGT-3′; *β-tubulin*, 5′-TGATGTGCGCAAGTGATCC-3′ and 5′-TCCTTTGTGGACATTCTTCCTC-3′), and 4 mL of template DNA. Amplifications were performed under the following conditions: 94°C for 15 min, followed by 40 cycles of 94°C for 15 s, 58°C for 20 s, and 72°C for 20 s. The number of *Pfmdr-1* copies of each sample was measured in triplicate relative to a standard curve by using 4 standards of mixed plasmids cloned into TOPO cloning vector (Invitrogen, Saint Aubin, France): standard 1 (1:1 ratio of *Pfmdr-1* and β-tubulin), standard 2 (2:1 ratio of *Pfmdr-1* and *β-tubulin*), standard 3 (3:1 ratio of *Pfmdr-1* and *β-tubulin*) and standard 4 (4:1 ratio of *Pfmdr-1* and *β-tubulin*) and 2 parasite clonal lines used as controls, the 3D7 Africa line (1 copy of *Pfmdr-1*) and line Dd2 (3 copies of *Pfmdr-1*), by the ΔCT method (where CT is the cycle threshold). We defined >1.6 copies as a duplication of the gene.

Evaluation of the Number of *Pvmdr-1* Copies

We measured number of *Pvmdr-1* (*Pfmdr-1*) copies following the same procedure, relative to the single copy of the *β-tubulin*. Briefly, PCRs were conducted in 25-mL volumes in a 96-well plate containing 1X HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne), 0.3 mM of each forward and reverse primer (*Pvmdr-1*, 5′-GCAACTCCATAAAGAACAACATCA-3′ and 5′-TTTGAGAAGAAAAACCATCTTCG-3′; *β-tubulin*, 5′-CATGTTCGTTAAGATTTCCTGGT-3′ and 5′-GTTAGTGGTGCAAAACCAATCA-3′), and 4 mL of template DNA. Amplifications were performed under the following conditions: 94°C for 15 min, followed by 45 cycles of 94°C for 15 s, 59°C for 30 s, and 72°C for 30 s. The number of *Pvmdr-1* copies of each sample was measured in triplicate relative to a standard curve by using 6 standards of mixed plasmids cloned into TOPO cloning as described for *Pfmdr*-1 (from the standard-1, 1:1 ratio of *Pvmdr-1* and *β-tubulin* to the standard-6, 6:1 ratio of *Pvmdr-1* and *β-tubulin*) by the ΔCT method (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/10/14-0411-Techapp1.pdf). We defined >1.6 copies as a duplication of the gene.

Statistical Analysis

Microsoft Excel 2010 (Microsoft, Redmond, WA, USA) and MedCalc software (v9.1.0.1, Mariakerke, Belgium) were used for data analysis. Categorical variables were compared by χ^2 test, and continuous variables were compared by using the 1-way analysis of variance or Mann-Whitney U test. We considered p values <0.05 as significant.

Ethical Approval

We obtained ethics clearance for the samples used in this study from National Ethics Committee in Cambodia (Ministry of Health), in Madagascar (Ministry of Health), in Sudan (Ministry of Health), and in France (National Reference Center for Malaria). All patients or their parents/guardians provided informed written consent.

Results

Global Distribution of the Number of *Pvmdr-1* Copies

We collected and analyzed 607 *P. vivax* isolates from areas to which the parasite was endemic from a total of 492 residents (117 in South America;, 117 in Asia; and 258 in Africa) and 115 travelers from France to South America (41 travelers), Asia (60), or Africa (14). The number of *Pvmdr-1* copies ranged from 1 to 5 copies (mean 1.28, 95% CI 1.22–1.34; median 1.05 [interquartile range (IQR) 0.84–1.53]) and was distributed as follows: 75% isolates had 1 copy, 18% had 2 copies, 6% had 3 copies, 1.6% had 4 copies, and 0.4% had 5 copies. The frequency of *Pvmdr-1*–amplified isolates was significantly higher in samples from South America (83 [53%] of 158) than in samples from Asia (60 [34%] of 177, $p = 10^{-3}$) or Africa (11 [4.0%] of 272, $p<10^{-5}$). The mean number of *Pvmdr-1* copies was also higher in isolates from South America (1.8) than in isolates from Asia (1.3, p = 0.0007) or Africa (0.9, $p<10^{-5}$). Number of copies differed significantly between *P. vivax* isolates from residents and those from travelers. In South America, the proportion of isolates with >1 copy of *Pvmdr-1* was significantly lower in travelers (34% vs. 59%, odds ratio [OR] 0.4 [95% CI 0.2–0.8], p = 0.007); in Africa, this proportion was significantly higher in travelers (57% vs. 1%, OR 113, 95% CI 24–536, p<0.0001) (Tables 1, 2; Figure 1).

Amplification of *Pvmdr-1* in Isolates from Residents

Number of copies in isolates from residents differed significantly among continents (Table 3). The mean

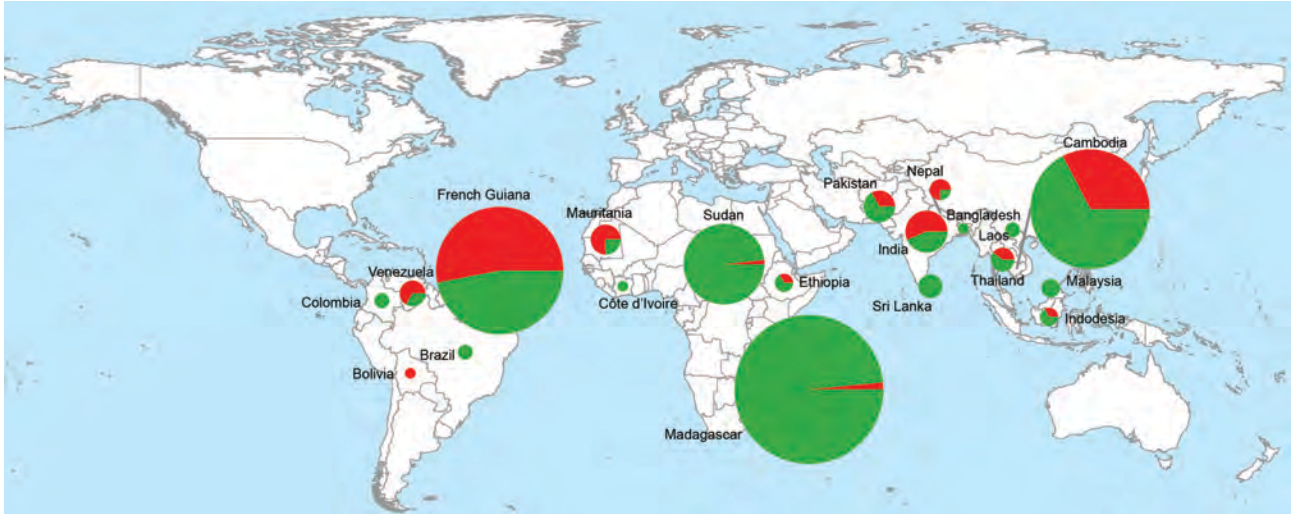

Figure 1. Geographic distribution of *Plasmodium vivax* isolates with 1 multidrug resistance-1 (*mdr-1)* copy (green) and isolates with >1 *mdr-1* copies (red) in 607 samples collected in South America, Asia, and Africa during 1997–2010.

Table 1. Variation in number of copies of *Plasmodium vivax mdr-1* gene in 607 isolates from residents of and travelers from France to malaria-endemic areas, South America, Asia, and Africa, 1997–2009

Variable	Residents	Travelers	Total	p value
No. isolates	492	115	607	–
Origin of isolate, no. (%)				
South America	117 (24)	41 (36)	158 (26)	–
Asia	117 (24)	60 (52)	177 (29)	
Africa	258 (52)	14 (12)	272 (45)	
Isolates with >1 *Pvmdr-1* copy				
No. (%; 95% CI)	109 (22; 18–26)	45 (39; 30–49)	154 (25; 22–29)	$<10^{-3}$*
Range	1–5	1–3	1–5	
Copies of *Pvmdr-1*, no. (%)				
1	383 (77.9)	70 (61)	453 (75)	$<10^{-8}$†
2	63 (12.8)	44 (38)	108 (18)	
3	34 (6.9)	1 (1)	35 (6)	
4	10 (2.0)	0	10 (1.6)	
5	2 (0.4)	0	2 (0.4)	
Mean (95% CI)	1.27 (1.21–1.34)	1.32 (1.22–1.41)	1.28 (1.22–1.34)	0.57‡

*χ^2 test.
†1-way analysis of variance.
‡Mann-Whitney U test.

number of *Pvmdr-1* copies was highest in isolates from South America (2.04, $p<10^{-5}$ vs. Asia and Africa), followed by Asia (1.32, $p<10^{-4}$ vs. Africa) and Africa (0.90 for Madagascar and 1.0 for Sudan). In South America, the proportion of isolates with >1 *Pvmdr-1* copies was also more frequent (59%) than in Asia (33%, OR 0.33, 95% CI 0.2–0.6, $p = 10^{-4}$) and Africa (1%, OR 0.008, 95% CI 0.002–0.02, $p<10^{-5}$ vs. South America; OR = 0.02, 95% CI 0.007–0.08, $p<10^{-4}$ vs. Asia).

Amplification of *Pvmdr-1* in Isolates from Travelers

The mean number of *Pvmdr-1* copies was similar for travelers returning to France from South America or Asia and did not change by year of collection: 1997–2000 (1.42 and 1.24, respectively), 2001–2005 (1.17 and 1.38), and 2006–2009 (1.20 and 1.49). In contrast, for travelers returning to France from Africa, the mean number of *Pvmdr-1* copies increased in samples collected more recently: 0.60 during 1997–2000, 1.02 during 2001–2005,

Table 2. Variation in number of copies of *Plasmodium vivax mdr-1* gene among isolates from residents of malaria-endemic continents and from travelers from France to those areas, 1997–2010

Continent	Residents	Travelers	Total	p value
South America				
Isolates with >1 *Pvmdr-1* copy	117	41	158	
No. (%; 95% CI)	69 (59; 50–68)	14 (34; 20–51)	83 (53; 44–60)	0.01*
Range	1–5	1–2	1–5	
Copies, no. (%)				
1	48 (41)	27 (66)	75 (47)	10^{-4}*
2	26 (22)	14 (34)	40 (25)	
3	32 (27)	0	32 (20)	
4	9 (8)	0	9 (6)	
5	2 (2)	0	2 (1)	
Mean (95% CI)	2.0 (1.8–2.2)	1.2 (1.0–1.3)	1.8 (1.7–2.0)	$<10^{-5}$†
Asia				
Isolates with >1 *Pvmdr-1* copy	117	60	177	
No. (%; 95% CI)	37 (32; 23–45)	23 (38; 24–57)	60 (34; 26–44)	0.5*
Range	1–4	1–3	1–4	
Copies, no. (%)				
1	80 (68)	37 (62)	117 (66)	0.68*
2	34 (29)	22 (37)	56 (32)	
3	2 (2)	1 (2)	3 (2)	
4	1 (1)	0	1 (1)	
Mean (95% CI)	1.3 (1.2–1.4)	1.4 (1.3–1.5)	1.3 (1.2–1.4)	0.41†
Africa				
Isolates with >1 *Pvmdr-1* copy	258	14	272	
No. (%; 95% CI)	3 (1; 0.2–3)	8 (57; 24–100)	11 (4; 2–7)	$<10^{-8}$*
Range	1–2	1–2	1–2	
Copies, no. (%)				
1	255 (99)	6 (43)	261 (96)	$<10^{-8}$*
2	3 (1)	8 (57)	11 (4)	
Mean (95% CI)	0.9 (0.9–1.0)	1.3 (1.1–1.6)	0.9 (0.9–1.0)	$<10^{-8}$†

*χ^2 test.
†1-way analysis of variance.

Table 3. Variation in number of copies of *Plasmodium vivax mdr-1* gene in isolates from residents of selected Asian and African countries

Variable	Country, years of sample collection				Total	p value
	French Guiana, 2000–2003	Cambodia, 2010	Madagascar, 2006–2007	Sudan, 2007		
No. isolates	117	117	199	59	492	
Isolates with >1 *Pvmdr-1* copy						
No. (%; 95% CI)	69 (59; 46–75)	37 (32; 23–45)	2 (1; 0.1–3.6)	1 (2; 0.4–9.5)	109 (22; 18–26)	$<10^{-10}$*
Range	1–5	1–4	1–2	1–2	1–5	
Copies, no. (%)						$<10^{-10}$*
1	48 (41)	80 (67)	198 (99)	57 (98)	383 (77.9)	
2	26 (22)	34 (30)	1 (1)	2 (2)	63 (12.8)	
3	32 (27)	2 (2)	0	0	34 (6.9)	
4	9 (7)	1 (1)	0	0	10 (2.0)	
5	2 (3)	0	0	0	2 (0.4)	
Mean (95% CI)	2.04 (1.85–2.24)	1.32 (1.22–1.43)	0.90 (0.84–0.91)	1.00 (0.94–1.05)	1.27 (1.21–1.34)	$<10^{-10}$†

*χ^2 test.
†1-way analysis of variance.

and 1.48 during 2006–2009 (OR 0.15, 95% CI 0.03–0.71, p = 0.049). The proportion of isolates with >1 copies of *Pvmdr-1* did not differ significantly among continents over time (Table 4).

Amplification of *Pvmdr-1* and *Pfmdr-1* in Isolates from Cambodia and Malagasy

We compared the distribution profile of the number of *mdr-1* copies of *P. falciparum* and *P. vivax* isolates in 2 different settings. In both Cambodia and Madagascar, the number of *mdr-1* copies did not differ between the 2 species (Figure 2). In Cambodia, where mefloquine has been widely used for >25 years, the mean of number of *mdr-1* copies was 1.34 for *P. falciparum* (n = 88, 95% CI 1.22–1.47, range 1–3) and 1.34 for *P. vivax* (n = 129, 95% CI 1.24–1.44, range 1–4, p = 0.52). In contrast, in Madagascar where mefloquine has never been recommended and has been barely used, the mean number of *mdr-1* copies was 0.92 for *P. falciparum* (n = 350, 95% CI 0.90–0.94, range 1–2) and 0.90 for *P. vivax* (n = 201, 95% CI 0.87–0.93, range 1–2, p = 0.21).

Discussion

Developed in the 1970s at the US Department of Defense's Walter Reed Army Institute of Research as a synthetic analog of quinine (*25*), mefloquine was introduced in 1983 in Thailand to replace chloroquine as first-line treatment for falciparum malaria (*26*). Since then, mefloquine alone or in combination with artesunate has been widely used, especially in Southeast Asia (including Cambodia) and South America (including French Guiana), where it was introduced for second-line treatment and for chemoprophylaxis in 1990 (*27*). In contrast, mefloquine has not been used extensively in Africa and has not been introduced in Madagascar. Mefloquine has been available for malaria chemoprophylaxis since 1985 in Europe and since 1990 in the United States and has been used by >35 million travelers from France for this indication (*28*,*29*).

Pfmdr-1 gene amplification has been described as the major mechanism of *P. falciparum* mefloquine resistance associated with treatment failure or in vitro resistance (*13*–*16*). Previous studies, including ours, confirm that *mdr-1* amplification does occur in *P. vivax (17–22)*. In addition, the epidemiologic data in our current study show that in regions where mefloquine has never been used, such as in Madagascar and Sudan, amplification in *Pvmdr*-1 is rare (1% and 2% of total isolates, respectively), whereas in areas with current or past intense use of mefloquine, such as in French Guiana and Cambodia, *Pvmdr*-1 amplification is frequent and detected in 59% and 33% of isolates, respectively, and with a mean of 2 and 1.3 copies, respectively.

Table 4. Variation in number of copies of *Plasmodium vivax mdr-1* gene among isolates from persons from France who reported having traveled during the previous month to malaria-endemic continents, 1997–2010

Variable	South America	Asia	Africa	Total	p value
No. isolates	41	60	14	115	
Isolates with >1 *Pvmdr-1* copy					
No. (%; 95% CI)	14 (34; 18–57)	23 (38; 24–57)	8 (57; 24–100)	45 (39; 30–49)	0.30*
Range	1–2	1–3	1–2	1–3	
Copies, no. (%)					0.49*
1	26 (63)	37 (62)	6 (43)	70 (61)	
2	14 (34)	22 (37)	8 (57)	44 (38)	
3	0	1 (2)	0	1 (1)	
Mean (95% CI)	1.20 (1.05–1.35)	1.40 (1.27–1.53)	1.35 (1.10–1.59)	1.32 (1.22–1.41)	0.12†

*χ^2 test.
†1-way analysis of variance.

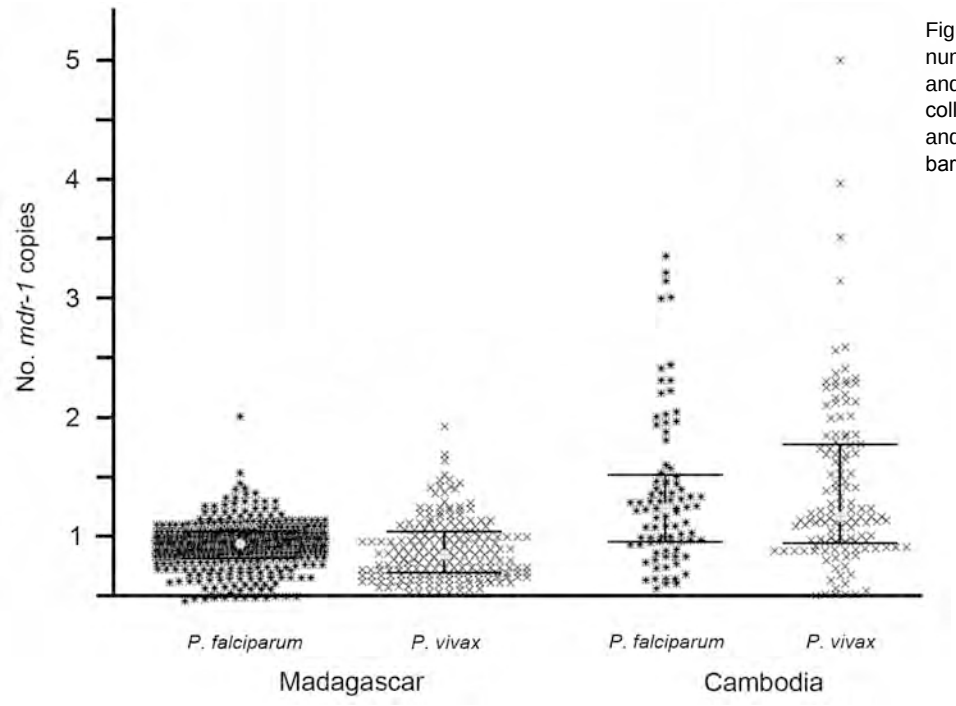

Figure 2. Comparative distribution of numbers of P*lasmodium falciparum* and *P. vivax* mdr-1 copies in isolates collected from residents of Madagascar and Cambodia. Gray dot, median; dark bars, interquartile range (25%–75%).

Both the number of copies and prevalence of *Pvmdr*-1 of isolates with multiple *mdr*-1 copies we observed here are much higher than reported in other studies. For instance, Imwong et al. reported *Pvmdr-1* amplification in 6/66, 2/50, and 1/49 isolates from Thailand, Laos, and Myanmar, respectively (*17*); Jovel et al. observed *Pvmdr-1* amplification in 1/37 in Honduras (*20*); and Lin et al. recently reported 39% and only 4% prevalence in *P. vivax* isolates from Thailand and Cambodia, respectively (*18*). A reason for the discrepancy between observations from Lin et al. in Cambodia and our observations could be because their isolates were collected during 2006–2007 and our samples were collected in 2010, indicating an increase in *Pvmdr-1* amplification over 3 years. Another reason is the location of collection. Indeed, drug resistance and drug pressure markedly differ across Cambodia. Lin et al. studied isolates from southern Cambodia (Kampot Province), whereas we studied isolates from areas in which drugs were highly resistant in western (Pailin Province) and southeastern Cambodia (Kratie Province), where multidrug resistance of *P falciparum* is emerging (*30*) and drug pressure (including artesunate–mefloquine combination) has been intense in recent years. To our knowledge, the previous maximum number of *Pvmdr-1* copies detected was 3 (*18*,*19*); in this study. however, we observed up to 5 copies in isolates from South America and 4 copies in isolates from Southeast Asia. This difference is likely to be due to our real-time PCR approach using 6 standards of mixed plasmids, which enabled detection of *P. vivax* isolates with *mdr-1* amplification with up to 6 copies. This observation also could indicate an ongoing selection of *mdr-1*–amplified parasites. Although, our data need to be confirmed and supported by in vivo data from mefloquine-treated patients or in vitro experiments showing a direct relationship between mefloquine pressure and *P. vivax mdr-1* amplification, our findings advocate for an integrated drug policy whereby all sympatric malaria species are considered regarding treatment efficacy but also drug pressure and selection of resistance.

We were able to assess the number of *Pvmdr-1* copies in isolates collected from travelers returning to France. These data must be analyzed with caution because information about the location of infection might be erroneous, particularly given the fact that relapses from hypnozoites can occur several months after primary infection. We cannot exclude that a patient declaring having returned from Africa was previously infected during a trip to a different location because we did not include this information in the questionnaire administered at recruitment. Nevertheless, we found significant differences between travelers from France and residents from a given geographic origin, especially in isolates from Africa, where most (99%) isolates from residents displayed no amplification, whereas most (57%) isolates from travelers from France had *Pvmdr-1* amplification. We assume this indicates within-host selection by mefloquine prophylaxis, which has been and continues to be widely used among travelers from France who go to malaria-endemic countries. Such pressure does not exist in residents, who usually do not take any prophylaxis.

These data are of concern because they suggest that selection of *Pvmdr-1* amplification is a more rapid process than previously thought, reminiscent to atovaquone resistance. Although selection in travelers from France returning to nonendemic areas bears no transmission risk, chemoprophylaxis and intermittent preventive treatments in malaria-endemic areas might contribute to the emergence of resistant parasites. This possibility certainly warrants further investigation.

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Ms Khim is a PhD student at the Malaria Molecular Epidemiology Unit in Institut Pasteur in Cambodia. Her research interests include clinical epidemiology and antimalarial drug resistance in vivax malaria and more generally are focused on developing molecular tools for improving the surveillance of resistance to antimalarial drugs in Cambodia.

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Risk Factors for Human Lice and Bartonellosis among the Homeless, San Francisco, California, USA

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Learning Objectives

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

- Distinguish features of trench fever
- Evaluate variables associated with head lice infestation among homeless adults
- Analyze risk factors for body lice infestation among homeless adults
- Assess the prevalence of *Bartonella quintana* among head and body lice

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Homeless persons in San Francisco, California, USA, have been shown to have head and body lice infestations and *Bartonella quintana* infections. We surveyed a self-selected population of homeless persons in San Francisco to assess infestations of head and body lice, risks of having body lice, and presence of *B. quintana* in lice. A total of 203 persons who reported itching were surveyed during

2008-2010 and 2012: 60 (30%) had body lice, 10 (4.9%) had head lice, and 6 (3.0%) had both. *B. quintana* was detected in 10 (15.9%) of 63 body lice pools and in 6 (37.5%) of 16 head lice pools. Variables significantly associated ($p \leq 0.05$) with having body lice in this homeless population included male sex, African-American ethnicity, and sleeping outdoors. Our study findings suggest that specific segments of the homeless population would benefit from information on preventing body lice infestations and louse-borne diseases.

The human body louse (*Pediculus humanus humanus*) has played a key historical role in the transmission of diseases such as trench fever, epidemic typhus, and louse-borne relapsing fever (*1*,*2*). Because of the emergence of pesticide-resistant head lice (*P. humanus capitis*) (*3*), lice continue to be part of the human landscape. Head lice are not implicated in the transmission of disease-causing agents, but they do have social and economic effects because of the school days missed by children with infestations (*3*). Like head lice, pubic lice (*Pthirus pubis*) are not known to transmit pathogens (*4*). Although diseases are not transmitted by all lice species, heavy louse infestations can cause pruritus, secondary infections from scratching, and anemia (*5*,*6*).

A combination of conditions, including poor clothing hygiene, lack of resources, and cold weather, put certain human populations, such as homeless persons, more at risk of harboring lice and for louseborne diseases (*2*). Research suggests that environments frequented by homeless persons (e.g., homeless shelters, where there is close body-to-body contact and where clothing hygiene is lacking) promote louse transmission (*2*,*5*). The number of homeless persons is not trivial. A single night count by the Annual Homeless Assessment Report (AHAR) estimated 649,917 homeless persons in the United States in 2010; of that total, 5,823 (0.9%) were in San Francisco (*7*). Given the large number of homeless persons in the United States, human lice of all species and the diseases they may carry are a health concern for this population.

Of concern is the potential for body lice to transmit *Bartonella quintana*, the bacterium that causes trench fever, the most common louseborne disease in some urban homeless persons (*8*). Trench fever is characterized by severe frontal headache, dizziness, conjunctival congestion, shin pain, and lymphadenopathy, accompanied by a relapsing fever lasting 4–8 days at a time. Life-threatening complications, such as endocarditis and bacillary angiomatosis, can occur (*1*,*9*). The only known vector of *B. quintana* is the body louse, although recent studies suggest head lice may also vector disease agents (*10*–*12*). *B. quintana* proliferate in body lice 4 days after ingestion and are continuously excreted in feces for ≥3 weeks (*13*). *B. quintana* can be transmitted to a human host when an infected louse feeds on an uninfected human and excretes *B. quintana*-infected feces onto their skin (*1*). The bacteria are rubbed into open mucous membranes or scratched into the skin through the bite wound (*5*), resulting in *B. quintana* infection 15–25 days later (*1*). Although *B. quintana* are principally infective to humans, macaque monkeys and the lice that infest them can maintain and circulate a strain of *B. quintana* (*14*).

In a previous study of head and body lice from homeless persons in San Francisco, we determined that 33.3% of body lice samples and 25% of head lice samples, which were pooled by host, were positive for *B. quintana* (*12*). To better characterize body and head lice transmission and to gauge the risk for *B. quintana* infection, we evaluated San Francisco homeless persons to determine presence of head and body lice, assess habits that may increase the risk of acquiring lice, and collect lice to test for *B. quintana* infection.

Methods

Survey and Lice Collection

Methods for this study were reviewed and approved by the State of California Committee for the Protection of Human Subjects (protocol no. E-108-10-01). The California Department of Public Health, Vector-Borne Disease Section, participated in San Francisco's Project Homeless Connect (SFPHC), under the auspices of SFPHC medical services on 9 separate dates in 2008–2010 and 2012. In addition to providing the lice-related services described below, we administered a 15-question survey regarding lice transmission to all consenting homeless adults who inquired about services. Questions pertained to where the person slept, frequency of sharing/swapping used clothing, and prior exposure to lice. No personal identifiers were recorded. Persons came directly to the Vector-Borne Disease Section booth or by way of medical triage when they reported itching or "bug" problems. Persons requesting lice-related services received services regardless of whether they agreed to participate in the survey.

Lice-related services included examination of hair, body, and clothing for ectoparasites. Lice in the clothing or on the body below the neck were considered a positive indication of body lice infestation. Persons with body lice infestations were re-clothed to remove the source of lice. Those with infestations of more than a few lice were provided a shower and clean clothes. Infested persons were given verbal counseling on the control of body lice and a body lice fact sheet, and they were then accompanied to a licensed medical practitioner to complete their medical review.

Finding live lice on the head or hair, with the presence of nits, was considered a positive indication for a head lice

infestation. If a person had live head lice or >20 nits that were within a quarter inch of the scalp, they were given a kit with 1% permethrin lotion and a nit-remover comb, and were counseled on how to properly use the kit. Persons with severe head lice infestations or with matted hair were offered free haircuts.

The body and head lice that were collected from all persons were processed as previously described (*12*). In brief, ≤20 lice of each species were collected from each person and placed, by identifying number and species, into tubes with 70% ethanol. In the laboratory, both lice species were counted and placed into clean tubes with 70% ethanol; each person's pool contained 1–20 lice. For each person, only 1 pool of each louse species was tested. The lice pools were sent to the *Bartonella* Laboratory at the Centers for Disease Control and Prevention, Fort Collins, Colorado, USA, for DNA extraction, PCR testing, and sequencing.

Detection of *Bartonella* by PCR

Sixteen pools of head lice and 63 pools of body lice were homogenized by using a Mixer Mill MM 200 (Retsch, Newtown, PA, USA). The homogenate was prepared for DNA extraction by using a QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's tissue protocol.

PCR was performed in a TaKaRa PCR Thermal Cycler Dice (Takara Bio Inc., Shiga, Japan) targeting the citrate synthase gene (*gltA*) and the 16S-23S rRNA intergenic transcribed spacer region (ITS), using previously described primers (*15*,*16*). The PCR cycle conditions for *gltA* were 2 min at 95°C, followed by 38 cycles of 30 s at 95°C, 30 s at 48°C, 2 min at 72°C, and an extension for 7 min at 72°C. The conditions for ITS were 3 min at 95°C, followed by 55 cycles of 30 s at 95°C, 30 s at 66°C, 30 s at 72°C, and an extension for 7 min at 72°C. *B. doshiae* DNA was used as a positive control and nuclease-free water was used as a negative control.

The PCR amplicons were purified by using a QIAquick PCR Purification Kit (QIAGEN) and were sequenced on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence data were analyzed by using Lasergene 9 software (DNAstar, Madison, WI, USA) and aligned with *Bartonella* type strains from GenBank.

Data Analysis

All statistical analyses were conducted by using R statistical software (RStudio version 0.97.336; http://www.rstudio.com/); p values ≤0.05 were considered significant. The analysis included data from all persons who had a body lice examination and who agreed to participate in the survey. Persons were classified as having body lice or not having body lice, on the basis of the outcome of the examination. The explanatory variables analyzed in relation to body lice presence follow: reason for requesting services (bugs, itch, lice, or free shirt), sex, age, race/ethnicity, amount of time homeless (<1 year, 1–5 years, >5 years), where they slept (specific San Francisco district), whether they slept indoors or outdoors, frequency of sleeping in close proximity to others, frequency of clothing exchanged, and previous exposure to lice. We conducted univariate analysis, using χ^2, for each explanatory variable to test for association with the outcome of having body lice. Explanatory variables that were determined to have a significant association ($p \leq 0.05$) with a person having body lice were included in a generalized linear model to further analyze the predictive value of the variables for the outcome of having body lice. Having body lice or not was coded as a binary variable, and a logistic regression was run by using a backward process. The initial model contained all the significant variables from the univariate analysis. Variables were removed from the model, and their change effect was observed. The change in Akaike information criterion was also used as an indicator for the model's predictive value.

Survey participants co-infested with head and body lice (n = 6) were included in the body lice analysis. Data from the few persons with head lice only (n = 10) were analyzed separately, and a descriptive summary is provided.

Results

Survey

In all, 203 persons received an examination and completed a survey. Of these 203 persons, most (145, 71%) were men. The median age was 46 years (range 19–68). The racial/ethnic make-up was 91 (45%) white, 51 (25%) African American, 28 (14%) Hispanic, 24 (12%) Native American, and 9 (4%) Asian–Pacific Islander.

Survey participants reported spending most of their time in 19 different San Francisco districts or neighborhoods that are spread throughout the city; a 50-square-block area in north-central San Francisco (the Tenderloin district) was reported most frequently (76/203 reports, 37%). Of the 203 persons, 134 (66%) reported regularly sleeping indoors, including in shelters, single resident occupancies, and motels or other establishments; 65 (32%) reported sleeping outdoors in public locations; and 4 (3%) did not provide responses. Survey participants reported being homeless for <1 year (93, 46%), 1–5 years (53, 26%), or >5 years (36, 18%). Twelve (6%) of the 203 survey participants reported not being homeless, and 9 (4%) did not respond to the question. Surveys from these 21 persons were included in the analysis because, by participating in SFPHC, they were considered part of the homeless community.

Of the 203 survey participants, 132 (65%) reported never exchanging clothes with others, 35 (17%) reported

doing so once a month or "sometimes," 23 (11%) reported doing so ≥4 times a month or "frequently," 9 (5%) reported doing so 2–3 times a month or "often," and 4 (2%) did not respond. Of the 150 persons who responded to a question about sleeping distance from another person, 75 (37%) reported never sleeping within an arm's length of another person, and 75 (37%) reported always doing so. Slightly over half of the survey participants (107, 53%) reported previously having lice.

Head Lice

Ten of the study participants had head lice only, and 6 had head and body lice. Of the 10 with head lice only, the mean age was 46 years (range 22–58 years), 4 (40%) were male, 7 (70%) reported race/ethnicity as white and 3 (30%) as Native American, and all 10 reported having had lice before. Of the 10 persons with head lice only, 3 (30%) reported sleeping in the Tenderloin district, and 6 (60%) reported sleeping indoors, including at single resident occupancies and hotels. None of the 10 reported sleeping in a shelter. Three (30%) of the 10 persons reported never sleeping in close proximity to others, and 3 (30%) reported always sleeping in close proximity to others. Most of those with head lice only (7/10, 70%) reported never exchanging clothing.

Body Lice

Of the 203 persons who received a body lice examination, 60 (30%) were found to have body lice, including 6 who were co-infested with head lice. Table 1 shows a list of explanatory variables that were compared between persons with and those without body lice to determine an association with body lice infestation. Significant differences were found for sex, race/ethnicity, sleeping location, and the reason for the examination. Male sex, African-American ethnicity, sleeping outside, and lice being the reason for requesting services were more frequent among persons with body lice than among those without body lice (Table 1). In the final generalized linear model with the highest predictive value, the following were significantly associated with having body lice: male sex, African-American ethnicity, sleeping outdoors, and lice as the reason for requesting services (Table 2).

Lice Testing

Sixty pooled body lice samples, from 60 persons, were tested by PCR. In addition, 3 pooled samples from 3 persons without surveys were also tested. Of the 63 body lice pool samples, 10 (16%) were positive for *B. quintana*. Of the 7 surveyed persons with *B.* quintana–positive body lice, 6 (86%) were male, 5 (71%) were white, 1 (14%) was African American, and 1 (14%) was Native American. Of those 7 persons, 5 (71%) reported sleeping outdoors and 2 (29%) reported sleeping indoors.

Of 16 head lice pools, 6 (38%) were positive for *B. quintana*. All head lice samples came from persons who completed questionnaires. Of the 6 persons with positive samples, 4 (67%) were male, 5 (83%) were white, and 1 (17%) was Native American. Of those 6 persons, 4 (67%) reported sleeping outdoors and 2 (33%) reported sleeping indoors.

None of the persons with co-infestations (n = 6) had head and body lice positive for *B. quintana*. However, the head lice pool for 1 co-infested person was positive, and the body lice pool for another co-infested person was positive for *B. quintana*.

The B. quintana gltA sequences that we obtained were not distinguishable from a sequence previously submitted to GenBank (accession no. U28073). The ITS sequences that we obtained also were not distinguishable from a sequence previously submitted to GenBank (accession no. AF368391).

Discussion

By combining body lice examinations with a behavioral questionnaire in this study, we documented demographic and behavioral factors associated with an increased risk of acquiring body lice in this self-selected population of homeless persons. We found that 30% of persons in our study, most of whom sought services for possible lice infestation, had body lice. In other homeless populations, a body lice infestation prevalence of 7%–22% has been reported (*8*,*17*). However, the 16% pooled prevalence of *B. quintana* in the recovered lice in our study is similar to that in a previous study of a similarly selected group from the homeless population in San Francisco (*12*). This prevalence, along with the knowledge that body lice can transmit *B. quintana* (*8*), is of concern and suggests that bartonellosis (trench fever) is a risk among these homeless persons.

The results from our questionnaire suggest additional avenues of investigation to improve body lice prevention efforts. Male sex, African-American ethnicity, sleeping outdoors, and lice as reason for requesting services were significantly ($p \leq 0.05$) associated with having body lice in our study population of homeless persons (Table 2). Reasons such as inadequate access to resources or differences in risk behavior may explain the demographic factors that were significant (*18*). In a January 2012 point-in-time estimate, it was determined that 57.2% of the San Francisco homeless population was unsheltered, compared with 38% across the United States (*19*). We showed that persons sleeping outdoors had an increased risk for body lice. Several of the persons in this study who slept outdoors noted having lice-infested sleeping bags. Studies conducted in France found that shared bedding in shelters is a key factor in lice transmission (*9*,*17*). Thus, the potential for louse

Table 1. Association of body louse infestation and explanatory variables in a homeless population surveyed during 2008–2010 and 2012, San Francisco, California, USA

Variable	No. (%) with body lice (n = 60)	No. (%) without body lice (n = 143)	p value*
Sex			0.00†
Male	55 (91.7)	90 (62.9)	
Female	5 (8.3)	52 (36.4)	
No response	0	1 (0.7)	
Race/ethnicity			0.05†
White	27 (45.0)	64 (44.8)	
African American	21 (35.0)	30 (21.0)	
Hispanic	6 (10.0)	22 (15.4)	
Native American	4 (6.7)	20 (14.0)	
Asian	2 (3.3)	7 (4.9)	
Age group			0.19
19–28 y	1 (1.7)	11 (7.7)	
29–38 y	7 (11.7)	20 (14.0)	
39–48 y	27 (45.0)	43 (30.1)	
49–58 y	19 (31.7)	54 (37.8)	
59–68 y	5 (8.3)	15 (10.5)	
No response	1 (1.7)	0	
Sleeping			0.01†
Inside	30 (50.0)	104 (72.7)	
Outside	30 (50.0)	38 (26.6)	
No response	0	1 (0.7)	
Exchange clothing			0.76
Frequently	6 (10.0)	17 (11.9)	
Often	3 (5.0)	6 (4.2)	
Sometimes	8 (13.3)	27 (18.9)	
Never	41 (68.3)	91 (63.6)	
No response	2 (3.3)	2 (1.4)	
Time homeless			0.97
<1 y	29 (48.3)	64 (44.8)	
1–5 y	15 (25.0)	38 (26.6)	
>5 y	11 (18.3)	25 (17.5)	
Not homeless	3 (5.0)	9 (6.3)	
No response	2 (3.3)	7 (4.9)	
Reason for examination			0.02†
Bugs	5 (8.3)	28 (19.6)	
Itch	32 (53.3)	50 (35.0)	
Lice	18 (30.0)	37 (25.9)	
Services (free shirt)	5 (8.3)	28 (19.6)	

*p value of χ^2 test evaluating the distribution of variables between those with and without body lice.
†Indicates the distribution of the variable is significantly ($p \leq 0.05$) different between groups.

transmission through shared outdoor bedding (i.e., sleeping bags) warrants further investigation.

The length of time being homeless was not associated with body lice infestation in our study. This finding is similar to that in a previous study in which the amount of time a person had been homeless was not significantly associated with louse infestation among 126 homeless persons from emergency departments and shelters in Marseille, France (*20*). In the same study, Foucault et al. (*20*) found that the presence of body lice was associated with *B. quintana* bacteremia. Although we could not assess *B. quintana* infection in the persons in our study, we did document that the body lice from 10 (16%) of 63 body lice–infested persons were positive for *B. quintana*, suggesting an infection risk for these persons. Both studies underscore that lice infestations, and potential *B. quintana* infection, can occur at any time during a period of homelessness.

Persons who are unaware of a personal lice infestation may contribute to body lice transmission cycles in ways that have not been examined. In this study, there was no significant difference in lice infestation between those who reported sharing/swapping clothes and those who did not report such behavior. However, participants' answers to this question could be biased by a reluctance to admit wearing unwashed, used clothing. Persons came to our booth when they believed they had a problem, so we were not able to estimate the prevalence of lice infestation in the homeless population overall.

From the time *B. quintana* DNA was first detected in head lice, these lice have been of interest in studies of bartonellosis and of *B. quintana* transmission, but the epidemiologic role of head lice is still unclear (*10*, *12*,*21*). The percentage of *B. quintana*–positive head lice pools (37.5%) in the current study was slightly higher than the percentage we reported in 2007–2009 (22.2%) (*12*), but

Table 2. Results of logistic regression for the association of body louse infestation with certain explanatory variables in a homeless population surveyed during 2008–2010 and 2012, San Francisco, California, USA

Coefficient	Estimated values	SE	p value	Odds ratio	95% CI
Intercept	−3.82	0.73	<0.01	Reference*	–
Sleeping outdoors	0.98	0.37	0.01	2.66	1.29–5.59
African American†	1.03	0.40	0.01	2.81	1.30–6.23
Male	1.73	0.52	0.00	5.64	2.19–17.57
Reason for requesting services, lice‡	1.22	0.60	0.04	3.39	1.09–1.21

*Reference group is female, who sleep indoors, of ethnic self-reported background other than African American, and who requested services for reasons other than body lice.
†All other races/ethnicities combined (white, Hispanic, native American, and Asian) was used as the reference group for this variable on the basis of the results of the univariate analysis
‡All other reasons for requesting services (bugs, itch, and services, such as t-shirts) was used as the reference group for this variable on the basis of the results of the univariate analysis

the difference was not statistically significant. *B. quintana* has been detected from head lice collected from street beggars in Ethiopia (9.2%) and from street children in Nepal (9.5%), although some of the reported head lice infections in those studies may have been due to co-infestations of body and head lice (*10*,*21*). The presence of *B. quintana* in head lice and its absence in body lice (and vice-versa) from the same person may be due to the phenomenon of niche-sharing. In a recent genetic study of head and body lice collected from homeless persons (*22*), it was hypothesized that in cases of massive infestations of body and head lice, the lice will wander into the ecologic niches of the opposing lice, possibly evolving to colonize the new niche (*22*,*23*). Although we tried to collect lice that were clearly regionalized to the head (above the neck and with hair nits) or body (below the neck with clothing nits), the 2 types of lice could have migrated between regions and thus have been misclassified as body lice and vice versa. In our study, 6 persons had co-infestations of head and body lice, but none of these persons had both body and head lice positive for *B. quintana*. Further studies on body and head lice should genetically characterize lice species.

The nature of this study presented several limitations. First, our analysis and results are only generalizable to persons seeking lice-related services and may or may not be applicable to other members of the homeless community. Second, in several instances, we performed examinations of and collected lice from persons who did not participate in the survey but who had lice positive for *B. quintana*. Information for these persons was not available for the analysis conducted in this study, and such data would have further enhanced our understanding of the risk for *B. quintana* infection and the risk of acquiring lice. Third, although *B. quintana*–infected head lice were identified (37.5%), a small sample size precluded conclusions about the risk of acquiring head lice in this group or the role head lice may play in bartonellosis epidemiology. Last, the study protocol did not include the collection and testing of serum samples from survey participants, so we could not assess the risk that the presence of body lice, let alone the presence of infected body lice, specifically poses to human infection with *B. quintana*. Nonetheless, results from this study can influence public outreach messaging that sleeping outdoors is a risk behavior for the acquisition of body lice among the homeless. Although we did not identify a specific behavior associated with outdoor sleeping that could transmit lice, shared bedding (i.e., sleeping bags) may be a factor in the transmission of body lice for homeless persons who sleep outdoors.

The results from our study showed a relatively high prevalence of body lice (30%) in this group of homeless persons who self-selected for lice-related services. In addition, 16% of the recovered lice were infected with *B. quintana*, suggesting that this population is at risk for bartonellosis. The presence of body lice was positively associated with male sex, African-American ethnicity, and sleeping outdoors. Our findings suggest that focusing prevention information, such as promoting use of clean sleeping bags or explaining how to clean bedding, to those who sleep outside may be of additional benefit for decreasing lice infestations, and this possibility warrants further investigation.

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Influenza-Associated Hospitalizations, Singapore, 2004–2008 and 2010–2012

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Studies of influenza-associated hospitalizations in tropical settings are lacking. To increase understanding of the effect of influenza in Singapore, we estimated the age-specific influenza-associated hospitalizations for pneumonia and influenza during 2004–2008 and 2010–2012. The rate of hospitalization was 28.3/100,000 person-years during 2004–2008 and 29.6/100,000 person-years during 2010–2012. The age-specific influenza-associated hospitalization rates followed a J-shaped pattern: rates in persons ≥75 years of age and in children <6 months of age were >47 times and >26 times higher, respectively, than those for persons 25–44 years of age. Across all ages during these 2 study periods, ≈12% of the hospitalizations for pneumonia and influenza were attributable to influenza. The rates and proportions of hospitalizations attributable to influenza, particularly among the very young and the elderly, are considerable in Singapore and highlight the importance of vaccination in protecting populations at risk.

Seasonal influenza causes a substantial burden of illness worldwide. Infections can lead to severe illness that requires hospital care and can occasionally lead to death. Several studies have documented influenza-associated hospitalizations in countries with primarily temperate climates, such as the United States (*1*–*5*), and others have documented influenza-associated hospitalizations in subtropical regions, mostly in Hong Kong, China (*6*–*8*).

In the tropics, the spread of influenza is different from that in temperate regions because of the unique tropical climate and lack of clear climatic seasons (*9*,*10*). The baseline incidence of influenza infection is high, and >1 seasonal epidemic occurs each year (*11*). As documented in studies on influenza-associated deaths in Singapore (*9*,*12*), the effect of influenza epidemics in the tropics is comparable to its effect in other climatic regions. However, studies on influenza-associated hospitalizations in tropical settings are lacking. Such studies can provide an understanding of the pattern of hospitalizations and severe illness that is valuable in guiding public health policies.

Laboratory testing of specimens and virologic confirmation of influenza virus infections are not typically conducted for all patients and deaths; thus, the estimation of illness attributable to influenza cannot be based on reported episodes alone. Influenza can precipitate or exacerbate other respiratory and circulatory conditions, and there is a wide spectrum of clinical pathways and outcomes for influenza-associated conditions and complications. Pneumonia was ranked fifth in the list of top 10 conditions for hospitalization in Singapore in 2012 (*13*). There is a need to estimate the effect of influenza on health care utilization in terms of hospitalization for pneumonia and influenza.

Singapore has a robust data collection system, which facilitates the integration of databases from virologic surveillance for influenza and hospital systems. The aim of our study was to examine the influenza-associated hospitalization rates and proportions of pneumonia and influenza hospitalizations in Singapore. Age groups spanning <6 months to ≥75 years of age were examined to further identify the populations at greatest risk for influenza-associated hospitalizations.

Materials and Methods

Data

Singapore is a tropical city-state; the 2012 mid-year population was ≈5.3 million (*14*). Inpatient information from all hospitals in Singapore is captured in electronic medical records that include discharge diagnoses based on the 9th and 10th revisions of the International Classification of Diseases (ICD).

We obtained the weekly number of hospital admissions for principal discharge diagnosis of pneumonia and influenza (ICD-9 480–487 and ICD-10 J10–J18) during 2004–2008 and 2010–2012. We excluded data for 2009 because

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during the influenza A(H1N1)pdm09 pandemic that year, many persons were hospitalized for isolation purposes rather than on the basis of the clinical severity of illness. Ten groupings, by age, were considered: <6 months, 6–23 months, 2–4 years, 5–14 years, 15–24 years, 25–44 years, 45–64 years, 65–74 years, ≥75 years, and all ages.

The Ministry of Health (MOH), Singapore, has a national surveillance program for influenza, which was enhanced after the influenza A(H1N1)pdm09 pandemic. Before epidemiologic week 22 in 2009, virologic surveillance was based on diagnostic respiratory specimens of outpatients and inpatients from public acute-care hospitals, and subtyping was conducted on selected specimens. These specimens were tested either with informed consent from patients for diagnostic purposes or as part of epidemiologic surveillance provided for by the Infectious Diseases Act (*12*). Influenza viruses were identified by direct antigen detection using immunofluorescence techniques, serologic tests with complement fixation, and virus isolation. Beginning in epidemiologic week 22 in 2009, the specimens were obtained from outpatients with influenza-like illness (ILI), and all influenza virus–positive specimens were subtyped. Under the revamped sentinel surveillance program, nasopharyngeal and/or throat swab specimens were obtained from outpatients with ILI (temperature >38°C plus cough or sore throat) at government primary care clinics and private general practitioner clinics for influenza virus subtyping. Real-time reverse transcription PCR was used to determine influenza virus types and subtypes. Because of the change in surveillance sampling, we separately analyzed pre-2009 and post-2009 data.

Statistical Analysis

We chose the negative binomial regression model over the Poisson regression model after using a likelihood ratio test to test the model assumption; the results demonstrated that the negative binomial regression model was a better fit with the data. For each age group and the all-age group, we fitted the following negative binomial regression model to the weekly number of hospital admissions for pneumonia and influenza: weekly number of hospitalizations = long term trend and seasonality + influenza + respiratory syncytial virus (RSV) + weekly mean temperature + weekly mean relative humidity

To estimate the effect of influenza, we entered the weekly proportions of influenza virus–positive specimens across all ages; these data were derived from virologic surveillance and comprised reports of influenza A(H3N2), seasonal influenza A(H1N1), A(H1N1)pdm09, and influenza B infections. In our models, we used the all-age rather than age-specific proportion of influenza-positive specimens because the latter would have resulted in too few specimens for statistical analysis from patients <5 and ≥65 years of age. In addition, laboratory testing could be skewed toward particular age groups (mainly adults), which could result in a poor fit with the observed data in underrepresented age groups.

Seasonal peaks in hospitalizations for pneumonia and influenza may also be attributable to RSV. Thus, to avoid overestimation of hospitalizations for pneumonia and influenza attributable to influenza, we included in our model the weekly proportion of diagnostic specimens with test results positive for RSV. The data on RSV were from 2 public acute-care hospitals with pediatric departments that routinely test for RSV, which is known to predominately affect young children. These 2 public acute-care hospitals covered ≈63%–68% of hospitalizations for children <15 years of age in Singapore.

We made adjustment for potential confounding by including meteorologic variables in the regression models. To control for long-term trend and seasonality, we used a natural cubic spline function (piecewise smoothing polynomials) for time. We also used a nonlinear function with a natural cubic spline for weekly mean temperature and weekly mean relative humidity.

We evaluated model validity by plotting partial autocorrelation functions, which indicated that the specifications of the studied models were adequate and, hence, autoregressive terms of residuals were not included. We used the Spearman rank correlation coefficient to compare the association between the weekly number of hospitalizations for pneumonia and influenza and the proportions of specimens with test results positive for influenza virus in the 2 study periods.

Influenza-Associated Hospitalizations

The number of influenza-associated hospitalizations for pneumonia and influenza was defined as the sum of differences between the observed and expected weekly hospitalization numbers for pneumonia and influenza when influenza proportions were set to zero in the model (i.e., excess number attributable to influenza). We estimated the proportion of influenza-associated hospitalizations for pneumonia and influenza by dividing the total number of excess hospitalizations by the total number of observed hospitalizations.

The 95% CI for each estimated proportion was obtained by using the bootstrap resampling method with 1,000 resamples. The 2.5% and 97.5% quantiles of the 1,000 estimates were taken as the lower and upper bounds, respectively. The 95% CI for the number of influenza-associated hospitalizations for pneumonia and influenza was then derived by multiplying the number of observed hospitalizations for pneumonia and influenza by the respective 95% CI for the proportion of influenza-associated hospitalizations. The influenza-associated hospitalization rate per 100,000

person-years was obtained by dividing the total number of excess hospitalizations for pneumonia and influenza by the sum of the annual mid-year population estimates in the entire study period. The R statistical package, v3.0.0 (http://cran.r-project.org/bin/windows/base/old/3.0.0/) was used for analysis.

Results

During 2004–2008, a total of 59,519 diagnostic specimens were tested for influenza virus (Table 1). Over this 5-year period, there was an upward trend in the annual number of specimens tested, increasing from 13.1% of the total specimens in 2004 to 28.4% of the total specimens in 2008. A total of 3,131 (5.3%) specimens were positive for influenza virus. Among the influenza virus–positive specimens, a mean annual proportion of 72.2% (range 57.0%–86.4%) were positive for influenza A and 27.8% (range 13.6%–43.0%) for influenza B.

During 2010–2012, a total of 11,986 specimens from outpatients with ILI were tested for influenza virus. Over this 3-year period, there was a decreasing trend in the annual number of specimens tested; 58.2% of the total specimens were tested in 2010, compared with 17.6% in 2012. A total of 5,618 (46.9%) specimens were positive for influenza virus. Among the influenza virus–positive specimens, a mean annual proportion of 68.8% (range 52.0%–78.1%) were positive for influenza A virus, compared with 31.2% (range 21.9%–48.0%) for influenza B virus.

The higher percentage of influenza virus–positive specimens obtained during the second study period (46.9%), compared with percentage obtained during the first study period (5.3%), was due to the use of a more specific ILI definition for virologic surveillance and a more sensitive diagnostic method (i.e., reverse transcription PCR) during 2010–2012. For the first study period, the weekly number of hospitalizations for pneumonia and influenza and the proportion of influenza-positive diagnostic specimens were significantly correlated ($p<0.01$) (Figure 1). Likewise, for the second study period, the weekly number of hospitalizations for pneumonia and influenza and the proportion of influenza-positive specimens from outpatients with ILI were also significantly correlated ($p<0.01$) (Figure 1). The Spearman rank correlation coefficient was 0.587 during the first study period, compared with 0.314 during the second study period, when surveillance sampling was changed. These differences in correlation could be partly due to patient settings: during the first period, the specimens were obtained from patients in public acute-care hospitals, whereas during the second period, specimens were obtained from outpatients with ILI in community settings.

For all study years, except 2007, 2008, and 2011, the age-specific hospitalization rate per 100,000 person-years for pneumonia and influenza was lowest in the 25- to 44-year-old age group; for 2007, 2008, and 2011, the lowest rate was in the 15- to 24-year-old age group (Table 2). The annual hospitalization rate was consistently highest for persons ≥75 years of age.

In the 2 study periods, the age-specific influenza-associated hospitalization rates per 100,000 person-years for pneumonia and influenza showed a J-shaped pattern (Figure 2). The influenza-associated proportion was highest in children <6 months of age, and it was second highest in adolescents and young adults in the 15- to 24-year-old age group, followed by a decline in the older age groups in each of the 2 study periods.

During both study periods, the influenza-associated hospitalization rate per 100,000 person-years for pneumonia and influenza was highest among persons ≥75 years of age (452.2 and 458.9 during 2004–2008 and 2010–2012, respectively), second highest among children <6 months of age (213.9 and 363.6 during 2004–2008 and 2010–2012, respectively), and lowest among persons 25–44 years of age (8.1 and 9.7 during 2004–2008 and 2010–2012, respectively) (Table 3). The proportion of influenza-associated hospitalizations for pneumonia and influenza was highest among children <6 months of age (35.2% and 41.3% during 2004–2008 and 2010–2012, respectively). The proportion among persons 15–24 years of age was 25.1% during 2004–2008 and 23.6% during 2010–2012. The overall influenza-associated hospitalization rates per 100,000

Table 1. Virologic surveillance for influenza in Singapore during 2 study periods, 2004–2008 and 2010–2012

Study period	Total no. influenza–positive specimens/total no. tested	% Influenza–positive specimens	% Influenza A among influenza–positive specimens*	% Influenza B among influenza–positive specimens
Study period 1				
2004	294/7,783	3.8	67.0	33.0
2005	462/10,441	4.4	86.4	13.6
2006	535/11,105	4.4	72.9	27.1
2007	597/13,267	4.5	57.0	43.0
2008	1,243/16,923	7.3	77.6	22.4
Study period 2				
2010	3,461/6,971	49.6	78.1	21.9
2011	1,182/2,903	40.7	76.3	23.7
2012	975/2,112	46.2	52.0	48.0

*Includes all influenza A subtypes.

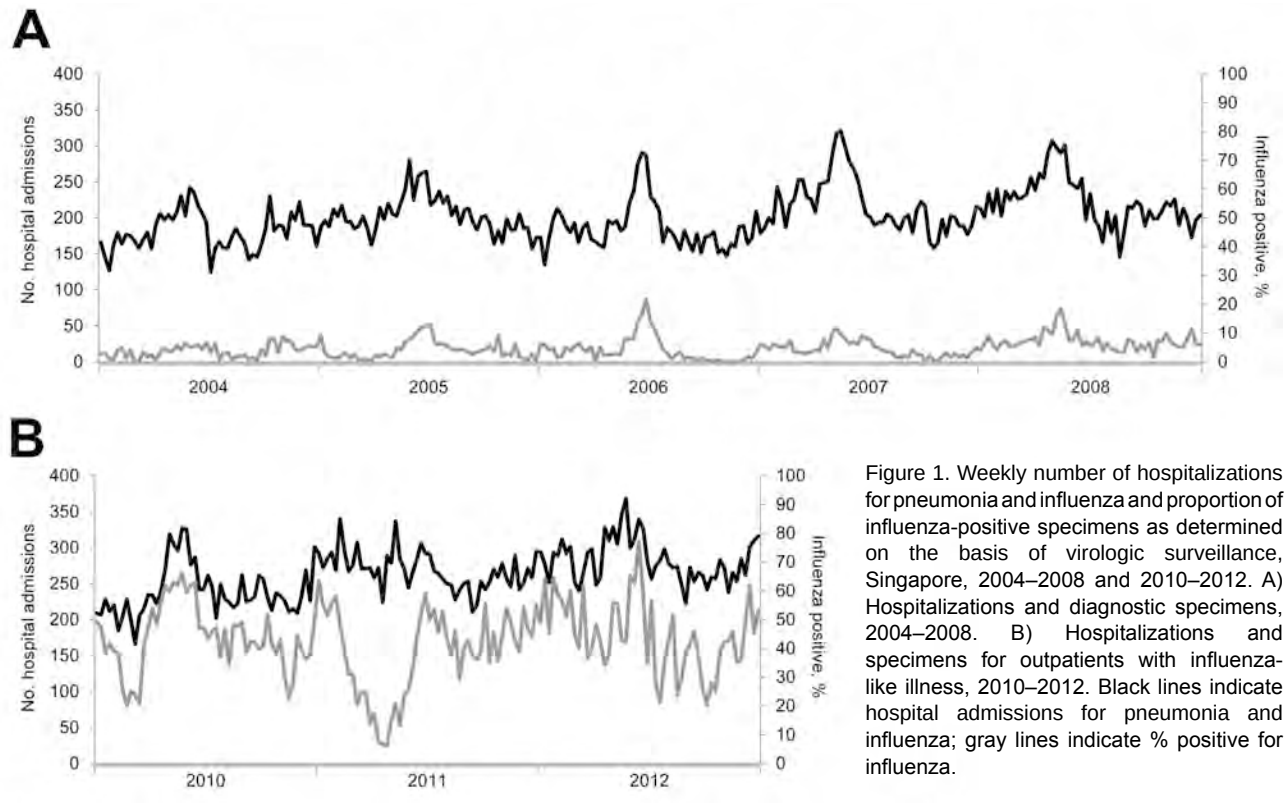

Figure 1. Weekly number of hospitalizations for pneumonia and influenza and proportion of influenza-positive specimens as determined on the basis of virologic surveillance, Singapore, 2004–2008 and 2010–2012. A) Hospitalizations and diagnostic specimens, 2004–2008. B) Hospitalizations and specimens for outpatients with influenza-like illness, 2010–2012. Black lines indicate hospital admissions for pneumonia and influenza; gray lines indicate % positive for influenza.

person-years (≈30) and proportions (12%) were similar during the 2 study periods.

The weekly proportion of diagnostic specimens with positive test results for RSV was statistically significant in the regression models for 2 age groups (<6 months and 6–23 months of age) in the first study period and 1 age group (5–14 years of age) in the second study period ($p<0.05$ in these 3 models). Sensitivity analyses showed that the estimates of influenza-associated hospitalization rates and proportions with and without RSV in regression models for all the age groups varied within 2%.

Discussion

Our analyses show that in Singapore, the age-specific influenza-associated hospitalization rates for pneumonia and influenza followed a J-shaped pattern that was also seen in analyses from other studies (*1*,*7*). The influenza-associated hospitalization rate was highest among persons ≥75 years of age, followed by children <6 months of age.

Our estimates of the number of influenza-associated hospitalizations in Singapore reflect the effect of influenza on the health care system in this tropical setting. The average length of hospital stay for pneumonia and influenza was ≈8 days. The mean annual estimate of influenza-associated hospitalizations for pneumonia and influenza during 2010–2012 was 1,535 (Table 3), which translates to 12,280 patient-days and a daily average of 34 occupied beds. We estimated that an additional 20 beds were occupied each day when influenza activity was at its peak. In the United States, studies estimating the national number of cases and hospitalizations averted by influenza vaccination support the use of influenza vaccination as a central tool for preventing influenza, and they highlight the need for increasing vaccination coverage and the need for more effective vaccines (*15*,*16*).

In Singapore, influenza vaccination is a key strategy for reducing the use of influenza-associated hospital services and influenza-associated illness and death. The Singapore MOH Expert Committee on Immunization recommended the use of influenza vaccine to protect vulnerable populations at higher risk for influenza-related complications; these populations include persons ≥65 years of age, adults and children with chronic medical conditions, pregnant women, and children 6 months to <5 years of age. Influenza vaccination has been shown to be cost-effective among the elderly and those with chronic medical conditions (*17*–*21*). Because the risk for influenza-associated hospitalization and death is high among the elderly and there may be lower effectiveness of influenza vaccine in the elderly (*22*–*25*), vaccination of household members of persons ≥65 years of age is encouraged to reduce transmission of influenza virus within the household (*26*–*30*).

In our study, children <6 months of age had the second highest age-specific influenza-associated hospitalization

Table 2. Age-specific hospitalization rates (per 100,000 person-years) for pneumonia and influenza in Singapore during 2 study periods, 2004–2008 and 2010–2012

	Hospitalization rates per 100,000 person-years							
	Study period 1					Study period 2		
Age group	2004	2005	2006	2007	2008	2010	2011	2012
0–5 mo	531.9	514.9	437.3	720.6	813.9	791.4	1107.4	751.8
6–23 mo	609.3	691.9	516.1	618.3	721.3	678.9	804.4	671.8
2–4 y	727.8	803.2	564.3	676.5	702.6	619.7	800.0	678.2
5–14 y	127.9	186.5	93.8	115.2	140.8	129.2	171.3	173.0
15–24 y	52.1	59.0	49.0	48.1	43.0	49.4	44.2	51.6
25–44 y	46.6	49.2	48.9	49.5	52.5	43.4	46.9	49.5
45–64 y	153.5	164.5	159.8	179.6	172.4	191.9	191.7	207.7
65–74 y	773.5	781.2	746.8	803.6	793.2	787.2	827.8	894.4
≥75 y	3,310.1	3,481.5	3,319.3	3,678.0	3,593.4	3,487.8	3,765.7	3,784.9
All	226.3	248.8	220.6	245.1	243.7	297.9	327.3	334.3

rate for pneumonia and influenza. Studies in different geographic regions (e.g., Finland, Hong Kong, and the United States) also found high hospitalization rates associated with influenza in children <1 year of age (*6*,*31*–*36*). Influenza vaccine is licensed only for use in persons ≥6 months of age. One way to reduce the risk of influenza in those <6 months of age is vaccination of their household contacts and caregivers, as recommended by the Advisory Committee on Immunization Practices in the United States (*30*).

Our findings underscore the importance of vaccination against influenza virus, in particular for the elderly, who are at higher risk for hospitalization and death. The Health Behavior Surveillance of Singapore, conducted by the Health Promotion Board by telephone and face-to-face with selected Singapore residents 18–69 years of age, showed that the proportion of persons who reported having been vaccinated against influenza in the preceding 12 months decreased significantly from 15.8% in 2010 to 11.2% in 2012 (p<0.001) (Health Promotion Board, unpub. data). In the 2012 Health Behavior Surveillance of Singapore, only 8.7% of adults 50–69 years of age reported having been vaccinated against influenza; this vaccine uptake percentage was half that for young adults 18–29 years of age (16.9%). In Hong Kong during the 2012–13 influenza season, vaccination coverage in the general population was 14.0%; the proportion of vaccinated persons was highest among persons ≥65 years of age (39.1%) and second highest among children 6 months to 5 years of age (28.4%) (*37*). In the United States during the 2011–12 influenza season, 44.3% of children 6–23 months of age received full vaccination, and 38.3% of persons ≥18 years of age were vaccinated (*38*). The National Health Interview Survey in the United States showed an increase in influenza vaccination coverage by age: 26.1% coverage among persons 18–49 years of age, 44.0% in persons 50–64 years of age, and 69.4% in persons ≥65 years of age.

Various measures have been implemented to raise awareness and increase influenza vaccine uptake in Singapore. For example, since 2014, the use of Medisave, a compulsory national health care savings scheme, has been allowed for payment of seasonal influenza vaccination in populations at high risk for influenza-associated hospitalization or death, including children 6 months to <5 years of age and persons ≥65 years of age. In addition, the Health Promotion Board and health care providers have made consistent efforts to educate the public on the importance of influenza vaccinations.

In Singapore, the proportion of influenza-associated hospitalizations for pneumonia and influenza was highest in children <6 months of age and second highest in the 15- to 24-year-old age group (Figure 2). There are many factors that may have affected this pattern; for example, hospitalizations for pneumonia and influenza attributable to viruses other than influenza, and the rate of testing by age group may have had an effect. In addition, there may be a higher propensity for testing and hospitalizing children <6 months of age compared with persons in older age groups.

This study in Singapore provides population-based estimates of influenza-associated hospitalizations for pneumonia and influenza, which enables comparison with estimates for other countries. In Singapore, the overall influenza-associated hospitalization rate per 100,000 person-years for pneumonia and influenza was 28.3 during 2004–2008 and 29.6 during 2010–2012. The proportion of influenza-associated hospitalizations was 11.9% and 11.2%, respectively, for the same years. A similar study in Hong Kong (a subtropical location), using Poisson regression based on data from 1996–2000, estimated that the influenza-associated hospitalization rate per 100,000 person-years for all ages was 29.3 and that 11.6% of all hospitalizations for pneumonia and influenza were attributable to influenza (*7*). In a study in the United States during 1979–2001, the overall rate of influenza-associated hospitalizations per 100,000 person-years for pneumonia and influenza was 36.8, and the proportion of influenza-associated hospitalizations was 8.6% (*1*).

In addition, during the 2 study periods (2004–2008 and 2010–2012) in our study, the rates and proportions of influenza-associated hospitalizations for pneumonia and influenza among the elderly (Table 3) were higher than those in Hong Kong during 1996–2000. In Hong Kong,

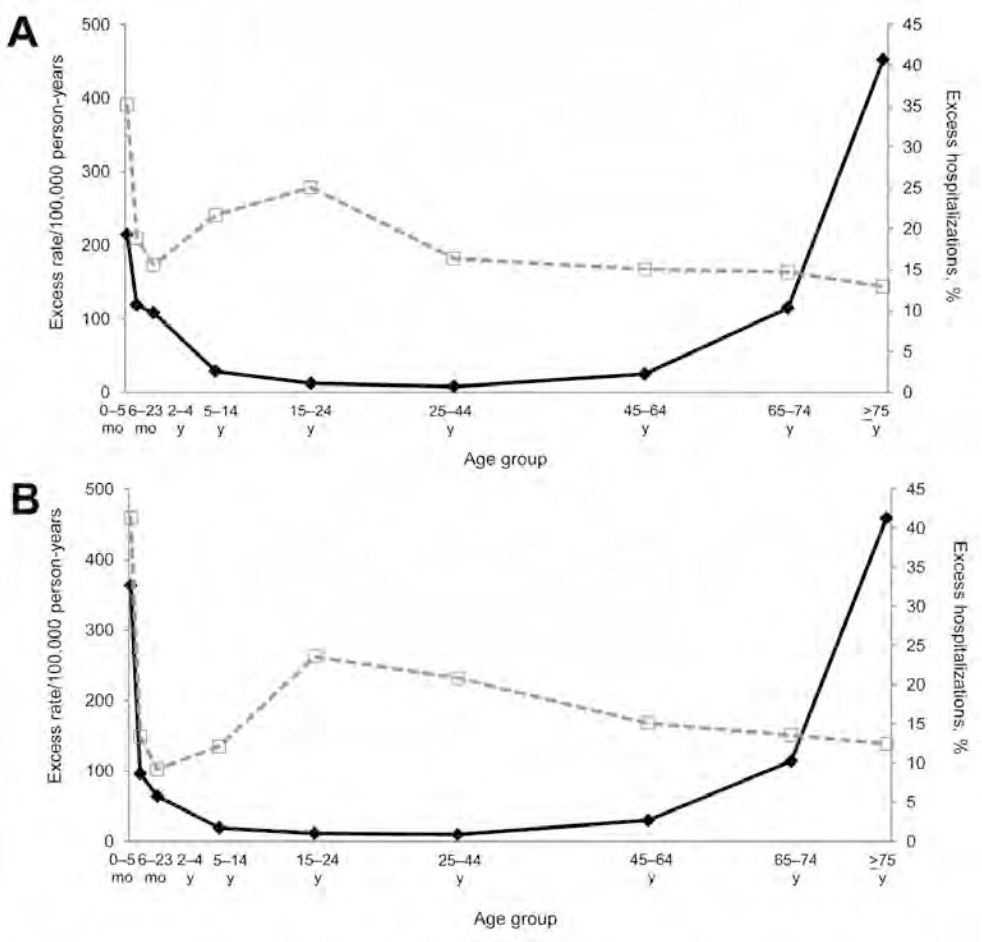

Figure 2. Age-specific rates (per 100,000 person-years) and proportions of influenza associated hospitalizations for pneumonia and influenza, Singapore. A) 2004–2008 and B) 2010–2012. Black bold lines indicate influenza-associated hospitalization rate per 100,000 person-years; gray dashed lines indicate % of influenza-associated hospitalizations.

the influenza-associated hospitalization rate per 100,000 person-years was 58.7 (95% CI 43.3–73.7) for persons 65–74 years of age and 176.3 (95% CI 119.2–231.0) for persons ≥75 years of age; the influenza-associated proportion was 11.0 (95% CI 8.1–13.8) and 7.1 (95% CI 4.8–9.3) in these 2 age groups, respectively (*7*). In Singapore, the influenza-associated hospitalization rate per 100,000 person-years among children <5 years of age was 92.3 (95% CI 74.1–118.3) during 2004–2008 and 65.8 (95% CI 36.5–114.6) during 2010–2012 (results not shown); these rates far exceeded the rate of 18.5 in the same age group in the United States during 1979–2001 (*1*). Various factors must be considered when comparing influenza-associated hospitalization rates and proportions by country. For example, comparisons should consider the age structure of the populations; influenza vaccination coverage, the level of herd immunity, and access to and utilization of health care services within the communities; the socioeconomic profile of the communities; and the climatic variables and severity of influenza epidemics in the communities. There are also variations in the statistical models, data aggregation, study periods, sampling protocols, and the coverage of laboratory virus surveillance systems.

Our study has several limitations. First, influenza-associated hospital admissions for pneumonia and influenza could be affected by many factors, such as uptake of influenza vaccines in the population, dominant influenza virus types/subtypes and their antigenic drifts and shifts, seasonal variations in vaccine match to circulating influenza strains, changes in admission criteria and diagnostic practices in hospitals, and variations in health care–seeking behavior. Second, although we controlled for potential confounding factors in our modeling approach, there may still be several unmeasured factors that could affect the estimates of influenza-associated hospitalizations for pneumonia and influenza. Third, in 2012 in Singapore, the diagnosis coding system for hospitals changed from ICD-9 to ICD-10; this change could have complicated the practice and assessment of cause-specific diagnoses. Last, our analysis was confined to hospitalizations for which the principal discharge diagnoses were pneumonia and influenza. However, during influenza seasons hospitalization rates increased for conditions other than pneumonia (e.g., acute bronchitis, chronic respiratory disease, and congestive heart failure) (*39*). In our study, we did not estimate the effect of hospitalizations for these other influenza-attributable conditions.

Table 3. Estimated influenza-associated hospitalizations for pneumonia and influenza in Singapore during 2 study periods, 2004–2008 and 2010–2012

Study period, patient age	Excess % (95% CI)	Excess no. per year (95% CI)	Excess no. per 100,000 person-years (95% CI)
Study period 1, 2004–2008			
0–5 mo	35.2 (27.3–41.8)	40 (31–48)	213.9 (166.1–253.9)
6–23 mo	18.9 (14.7–23.7)	74 (58–93)	119.1 (93.0–149.6)
2–4 y	15.6 (12.2–19.8)	141 (110–179)	108.3 (85.0–137.9)
5–14 y	21.7 (17.3–26.5)	150 (119–183)	28.8 (23.0–35.3)
15–24 y	25.1 (21.2–29.9)	87 (73–103)	12.5 (10.6–14.9)
25–44 y	16.4 (13.4–20.0)	139 (113–169)	8.1 (6.6–9.9)
45–64 y	15.1 (12.5–18.2)	251 (208–302)	25.1 (20.8–30.2)
65–74 y	14.7 (12.3–18.0)	238 (198–291)	114.9 (95.8–140.5)
≥75 y	13.0 (10.9–15.2)	518 (436–605)	452.2 (380.8–528.8)
All*	11.9 (10.4–13.8)	1,259 (1,100–1,456)	28.3 (24.7–32.7)
Study period 2, 2010–2012			
0–5 mo	41.3 (25.6–56.9)	68 (42–94)	363.6 (225.0–500.5)
6–23 mo	13.3 (6.7–23.4)	62 (31–108)	95.8 (48.3–168.3)
2–4 y	9.2 (4.6–16.7)	88 (44–160)	64.3 (32.4–117.2)
5–14 y	12.1 (6.0–21.5)	94 (47–166)	19.1 (9.5–33.8)
15–24 y	23.6 (12.7–38.2)	90 (49–146)	11.4 (6.2–18.5)
25–44 y	20.9 (12.3–30.5)	201 (118–294)	9.7 (5.7–14.2)
45–64 y	15.1 (9.1–22.0)	366 (220–535)	29.7 (17.9–43.5)
65–74 y	13.6 (8.2–19.9)	279 (168–409)	113.8 (68.6–167.0)
≥75 y	12.5 (7.8–18.0)	687 (431–993)	458.9 (288.3–663.8)
All*	11.2 (7.5–15.4)	1,535 (1,020–2,106)	29.6 (19.7–40.6)

*The excess number of hospitalizations was estimated from a model developed for each of the age groups and the all-age group. Thus, the number of excess hospitalizations in the all-age group was not the sum of excess hospitalizations across the 9 age groups.

Our findings allude to the importance of surveillance data for monitoring the effect of influenza and for assessing changes in influenza dynamics, as determined on the basis of a well-integrated virologic and epidemiologic surveillance system. For 2 reasons, we assumed that variation in influenza virologic surveillance by the MOH accurately reflected the patterns of influenza circulation in the general Singaporean population of Singapore: 1) during 2004–2008, the diagnostic respiratory specimens we used were from all public acute-care hospitals, and 2) the specimens during 2010–2012 were from outpatients with ILI at government primary care clinics and private general practitioner clinics that were geographically spread out across Singapore. In Singapore, 80% of the primary health care services are provided by private general practitioners; government clinics provide the remaining 20% of the primary health care services. The opposite is true for hospitalization care: public sector hospitalizations constitute 80% of all hospital admissions, and private sector hospitalizations constitute the remaining 20% (*40*). However, over time, there could be variation in laboratory testing patterns and sentinel sites for specimen collection in the community. Although we were unable to assess the effect of this variation, which may be somewhat reflected in the annual number of specimens tested, our modeling study was supported by sufficient year-round data for virologic surveillance.

Our findings have obvious policy implications. The J-shaped pattern observed for influenza-associated hospitalization rates during the 2 study periods was also seen in all of the individual years, both before and after the influenza A(H1N1)pdm09 pandemic. Our findings also underscore the importance of continuous surveillance in Singapore to identify populations at high risk for influenza-associated hospitalization or death and to guide public health policy priorities. The excess hospitalization estimates for pneumonia and influenza in our study reflect the considerable effect of influenza in Singapore, particularly among the rapidly aging population.

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Lyme Disease, Virginia, USA, 2000–2011

R. Jory Brinkerhoff, Will F. Gilliam, and David Gaines

Lyme disease, caused by the bacterium *Borrelia burgdorferi* and transmitted in the eastern United States by the black-legged tick (*Ixodes scapularis*), is increasing in incidence and expanding geographically. Recent environmental modeling based on extensive field collections of host-seeking *I. scapularis* ticks predicted a coastal distribution of ticks in mid-Atlantic states and an elevational limit of 510 m. However, human Lyme disease cases are increasing most dramatically at higher elevations in Virginia, a state where Lyme disease is rapidly emerging. Our goal was to explore the apparent incongruity, during 2000–2011, between human Lyme disease data and predicted and observed *I. scapularis* distribution. We found significantly higher densities of infected ticks at our highest elevation site than at lower elevation sites. We also found that *I. scapularis* ticks in Virginia are more closely related to northern than to southern tick populations. Clinicians and epidemiologists should be vigilant in light of the changing spatial distributions of risk.

Lyme disease (LD), caused by the bacterium *Borrelia burgdorferi* and transmitted in the eastern United States by the black-legged tick (*Ixodes scapularis*), is the most common vector-transmitted disease in North America (*1*). Maintained in an enzootic cycle comprising competent vertebrate reservoir host species, *B. burgdorferi* is transmitted to humans by the bite of an *I. scapularis* nymph or adult that acquired infection during a blood feeding as a nymph or larva (*2*). Although the principal reservoir host for this pathogen, the white-footed deer mouse, *Peromyscus leucopus*, is wildly distributed throughout North America, LD is generally confined to 2 geographic foci in the eastern United States: 1 in the upper Midwest and 1 in the Northeast (*2*–*5*). Densities of host-seeking *I. scapularis* nymphs correlate significantly with cases of human LD (*3*), but this species has been reported throughout much of eastern North America (*6*–*9*). Nationally, LD incidence increased during 1992–2002, but overall numbers of confirmed cases have since remained relatively stable (*1*,*10*).

In some locations, LD incidence recently has increased dramatically; in Virginia, the number of confirmed cases nearly tripled from 2006 to 2007 (http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm) to ≈12.4 cases per 100,000 residents, well above the 1998–2006 average of 2.2 per 100,000 (*1*). A 1990 report of LD cases in Virginia noted that the disease was rare in the early 1980s but apparently increased in incidence and geographic distribution through the late 1980s, leading the authors to conclude that the disease was expanding southward (*11*). Before 2006, most studies of *I. scapularis* ticks in Virginia focused on the eastern and southeastern parts of the state and found that densities of *I. scapularis* ticks declined, as did their rate of infection with *B. burgdorferi*, with distance from the coast (*12*,*13*). Several early surveys for *I. scapularis* ticks in Virginia's neighboring states of North Carolina and Maryland also found them to be most abundant on the Coastal Plain but absent or less common in the Piedmont and Appalachian Mountains. During 1983–1987, Apperson et al. surveyed 1,629 hunter-killed deer from the Coastal Plain, Piedmont, and Appalachian Mountain regions of North Carolina and found *I. scapularis* ticks only on deer from the Coastal Plain (*14*). Amerasinghe et al. surveyed 1,281, and 922 hunter-killed deer in 1989 and 1991, respectively, at sites from the Coastal Plain to the Appalachian Mountains of Maryland and found *I. scapularis* ticks on 59%–70% of deer on the Coastal Plain, fewer on deer in the Piedmont Region, and on only 1%–5% of deer in the Appalachian Mountains (*15*,*16*).

Although *I. scapularis* ticks exist in the southeastern United States (*6*–*9*), they are most easily detected by drag sampling, a method used as a proxy for risk to tick exposure (*5*), in areas associated with highest LD incidence, i.e., the Northeast (New Jersey through Massachusetts) and upper Midwest (Wisconsin and Minnesota) (*3*,*5*,*17*). The difference in apparent abundance of *I. scapularis* ticks and risk for LD between the northern and southeastern United States has been the subject of much discussion and debate (*18*) and might be related, either through behavioral or physiologic mechanisms, to genetic differences between *I. scapularis* populations in these regions (*7*,*19*–*22*). Population genetic

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structure of *I. scapularis* ticks has shown that dynamic range shifts are likely to have occurred in recent evolutionary history (*19*–*22*) and that 2 distinct lineages within this species can be identified; a relatively genetically uniform "American clade" exists in the northern United States (although this lineage has also been detected in the South), and a genetically diverse "southern clade," members of which have been found only in the South (*20*). Although other nomenclatures have been proposed for these 2 lineages (e.g., clades A and B for northern and southern lineages, respectively [*19*]), we follow the terminology established by Norris et al.: "American" describes the widely distributed yet less diverse clade and "southern" describes the geographically restricted yet more diverse mtDNA clade of *I. scapularis* ticks (*20*).

Range expansion of *I. scapularis* ticks over relatively short periods has been observed (*23*,*24*). Moreover, recent environmental modeling, based on extensive field collections of host-seeking *I. scapularis* ticks, suggests that this species suggests that the range of this species is expanding widely and its occurrence in a given area depends on the lack of abiotic drivers, vapor pressure deficit and elevation (*5*,*25*). In Virginia, studies found that *I. scapularis* ticks were concentrated in in northern sites; very few ticks were reported in other parts of the state (*5*,*17*,*25*). In contrast, human LD cases at inland, higher-elevation locations have increased in recent years in Virginia (http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm). The incongruity between human case and vector abundance datasets might be explained by recent (i.e., since 2007) spatial and/or numerical expansion of *I. scapularis* populations. We hypothesized that density of *B. burgdorferi*–infected ticks would be highest in counties associated with high incidence of human disease if epidemiologic data represent cases in tick-endemic areas. In contrast, low numbers of infected ticks in areas of high human disease might indicate either misdiagnosis or allochthonous exposure.

Methods

Data from Cases in Humans

We compiled LD cases reported to the Virginia Department of Health (http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm) directly by physicians or identified through follow-up of positive laboratory results by county public health department personnel during 2000–2011. All LD cases counted in Virginia State Reportable Disease Reports met clinical and laboratory criteria specified in the National Surveillance Case Definition (SCD) for LD. We assessed cases counted in Virginia during 2000–2007 using criteria in the 1996 SCD and cases counted during 2008–2011 using criteria in the 2008 SCD. The 1996 SCD enabled states to liberally interpret what constituted laboratory evidence of infection; an IgM-positive Western immunoblot (WB IgM) test result could be counted as laboratory evidence of infection even though a more specific 2-tier test that used an enzyme-linked immunoassay (EIA) and the WB IgM was recommended. The Virginia Department of Health used the less restrictive interpretation of laboratory evidence in its LD surveillance from 1996 through the end of 2007. However, given that single-tier positive results from either the EIA or the WB IgM are less specific than a positive 2-tier result from both the EIA and the WB IgM (*26*,*27*), laboratory evidence of infection in the 2008 SCD required, at a minimum, a positive 2-tier test result on blood collected during the acute phase of illness (i.e., within 30 days after illness onset). The more stringent laboratory criteria adopted in the 2008 SCD were designed to minimize the number of false cases counted by state surveillance programs.

We analyzed all data at the county level, which required us to reclassify cases reported in cities to the counties in which they are situated because cities and counties in Virginia are often separate administrative entities. We estimated LD incidence per county for each year during 2000–2011 by dividing the annual number of counted cases by the estimated population size in 2007 (*28*). To characterize annual change in incidence per county, we calculated the difference in cases between successive years and then averaged these values across years. We analyzed the spatial distribution of human LD cases at the state level by identifying the centroid, or geometric center, of county-level LD incidence for each year, starting in 2000 using ArcMAP 10.0 (ESRI, Redlands, CA, USA). We then used weighted linear regression to determine the effect of year on latitude and longitude of that year's centroid position weighted by annual number of cases.

Study Sites and Field Collections

In May and June 2011, we sampled ticks at 4 closed-canopy deciduous forest sites along an east-west elevational gradient: Crawfords State Forest (CR) (30 m), a University of Richmond–owned tract in Goochland County (GR) (80 m), Appomattox-Buckingham State Forest (AB) (170–200 m), and Lesesne State Forest (LE) (380–450 m) (Figure 1). We collected ticks at all sites by drag sampling (*29*) whereby a 1-m^2 piece of corduroy was dragged along both sides of 5 haphazardly selected 100-m transects (1,000 m^2 total), stopping every 20 m to remove ticks (*17*,*25*). We visited each site 4 times during May–July 2011 with at least 10 days separating visits. All ticks were speciated by light microscopy using dichotomous keys (*30*), and density of *I. scapularis* ticks was calculated as the average number of ticks collected per transect. Difference in density of *I. scapualris* nymphs among sites and visits was determined by analysis of variance of square root–transformed count

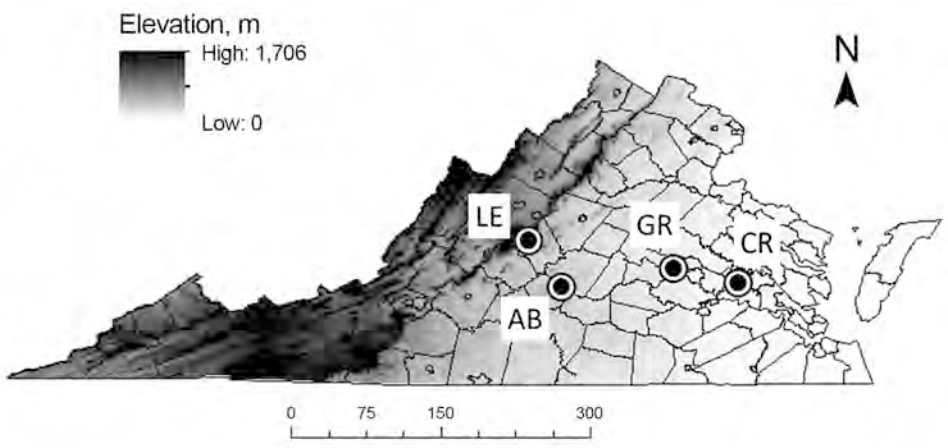

Figure 1. Locations of 4 field sites at which ticks were sampled, Virginia, May–July 2011. Circles indicate sampling areas. LE, Lesesne State Forest; AB, Appomattox-Buckingham State Forest; GR, University of Richmond–owned field site; CR, Crawfords State Forest. Darker shading represents higher elevation. Scale bar indicates kilometers.

data. We compared infection prevalence in ticks among sites by Gtest and by creating log-likelihood estimates of 95% CIs with a binomial probability function (*31*).

Molecular and Phylogenetic Methods

To extract total DNA, individual ticks were dried and flash-frozen by using liquid nitrogen, crushed by using a sterilized pestle, and processed with Qiagen DNeasy Blood and Animal Tissue Kit (QIAGEN, Valencia, CA, USA) by using manufacturer's protocols. We tested for *B. burgdorferi* DNA by PCR amplification of the outer surface protein C (*osp*C) gene and the intergenic spacer region of 16S–23S rRNA genes (*32*). Presence of amplified DNA was determined by gel electrophoresis, and samples that produced amplicons were purified with a QIAquick PCR Purification Kit (QIAGEN) and submitted for sequencing at the Nucleic Acids Research Facility at Virginia Commonwealth University (Richmond, VA, USA). We also performed PCR to amplify and subsequently sequence an ≈460-bp portion of the *I. scapularis* 16S rRNA gene using primers 16S +1 and 16S –1 (*20*). Bidirectional chromatograms from all sequence data were assembled and initially analyzed with Sequencher 4.10.1 (Gene Codes, Ann Arbor, MI, USA). *B. burgdorferi* sequences were blasted by using GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm species identification. Sequence data from *I. scapularis* 16S samples were aligned with reference sequences (*33*) by using ClustalW (http://www.clustal.org) implemented in MEGA 5.0 (http://www.megasoftware.net/), which was also used to select among models of evolution and to reconstruct phylogeny.

Results

During 1995–1998, the Virginia Department of Health counted 55–73 LD cases per year. The number increased to 122 cases in 1999, and cases continued to increase through the early 2000s. Although Virginia's LD activity during 2000–2005 was focused primarily on northern Virginia and the Eastern Shore of Virginia (a peninsula extending south from Maryland on the eastern side of the Chesapeake Bay), small numbers of LD cases were recorded in counties across Virginia, including counties in the most southern and southwestern parts (Figure 2). During 2006–2007 the incidence of LD increased substantially in counties throughout the Appalachian Mountains (Figure 2). After the change in the SCD in 2008, many of the most southern and southwestern counties that had recorded LD cases before 2008 ceased to report cases, and the geographic progression of LD appeared as a compact front that progressed from county to county from northeast to southwest. LD cases were not observed again in any of the far southwestern counties until 2011, by which time LD was considered endemic to many of the counties immediately to their northwest (Figure 2).

We collected 2,549 ticks from the field: 2,192 *Amblyomma americanum* (1 larva, 1,917 nymphs, 274 adults), 306 *I. scapularis* (304 nymphs, 2 adults), 50 *Dermacentor variabilis* (all adults), and 1 *I. dentatus* (nymph). Sampling site was a major determinant of *I. scapularis* density ($F = 71.07$, $p<0.0001$, degrees of freedom [df] = 3), as was sampling date ($F = 6.85$, $p=0.024$, df = 1). Post hoc comparisons indicated that tick density at the highest elevation site (9.55 nymphs/200 m^2) was significantly greater than at any other site and that tick density at GR (1.66 nymphs/200 m^2) was significantly higher than at site AB (0.25 nymphs/200 m^2) (Table). We detected *B. burgdorferi* DNA in 48 I. scapularis nymphs, 45 of which produced unambiguous sequence reads for at least 1 locus (*ospC* or intergenic spacer region. Infection prevalence varied significantly among sites (likelihood ratio test, $G = 16.3$, $p<0.0001$, df = 3); the prevalence of infection was significantly higher at site LE (0.2) than at sites CR (0.00) and GR (0.04). Because of low sample size, site AB did not yield a reliable estimate of infection prevalence (Figure 3).

Analysis of I. scapularis 16S sequences yielded 17 haplotypes (GenBank accession nos. KF146631–47) from

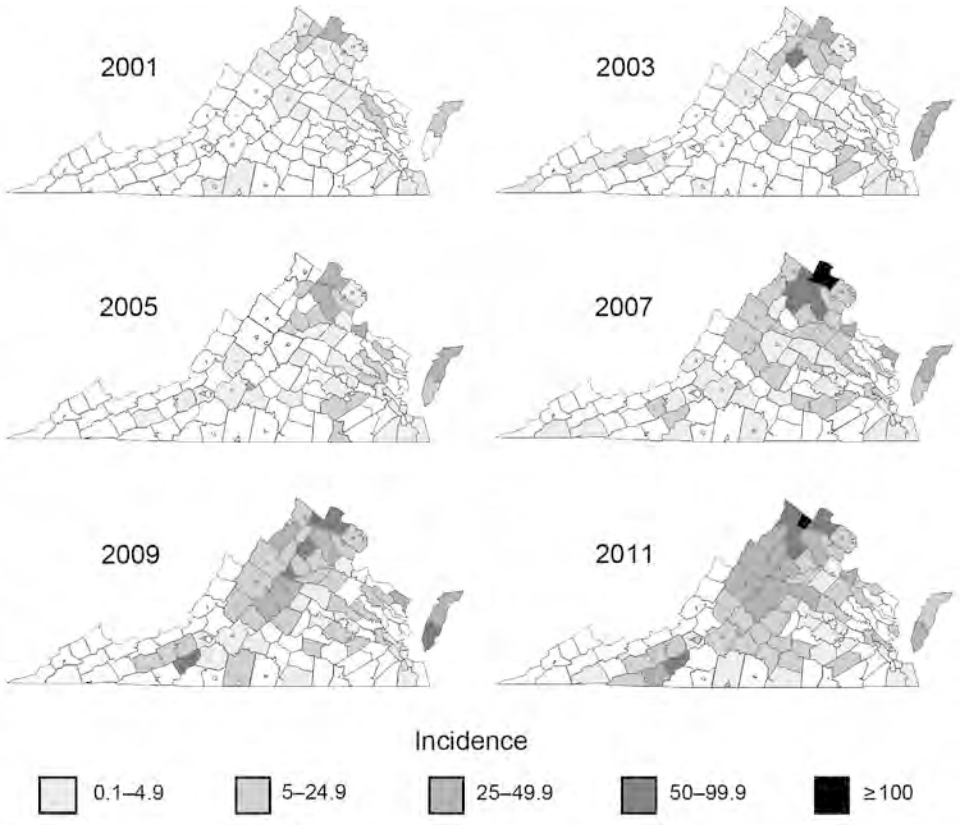

Figure 2. Progressive geographic spread of human Lyme disease across Virginia, 2001–2011. Data were reported by the Virginia Department of Health http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm. Cases per 100,000 population were calculated by county or city census estimate data published for the year preceding the year of the report.

85 individual nymphs (14 haplotypes from 44 ticks at LE, 1 from 2 ticks at AB, 6 from 21 ticks at GR, and 4 from 18 ticks at CR). Maximum-likelihood phylogenetic reconstruction using Tamura 3-parameter model (*34*) indicated that all haplotypes detected fall within the American clade; none of the ticks we sampled were phylogenetically identified as southern clade I. scapularis (Figure 4). In addition to an overall increase in human LD cases (from 136 in 2000 to an average of >1,000 in 2010 and 2011), we observed a significant spatial shift of the geometric center of LD incidence in Virginia. The longitude value associated with the centroid of each year's LD incidence depended significantly on year from 2000 to 2011 ($F = 12.48$, $p = 0.005$, $r^2 = 0.56$) (Figure 5). Latitude values did not change significantly over time ($F = 0.14$, $p = 0.71$, $r^2 = 0.01$). We also calculated the average LD incidence per county for 2000–2006 (before the dramatic spike in cases in Virginia) and for 2007–2011 to identify counties in which the largest increases in cases occurred (Table).

Discussion

Our results indicate that 1) human LD incidence in Virginia has increased since 2000 and that the spatial distribution of cases has changed significantly, 2) abundance of *I. scapularis* nymphs and prevalence of *B. burgdorferi* infection are consistent with recent changes in human disease data, and 3) *I. scapularis* populations detected in central and western Virginia are dominated by American-clade haplotypes. Taken together, these results suggest recent spatial and/or demographic expansion of *I. scapularis* ticks

Table. Average density of host-seeking *Ixodes scapularis* nymphs, average prevalence of *Borrelia burgdorferi*–infected nymphs, and average annual LD incidence in 5 counties, Virginia*

	Nymphs			Humans	
County	Average density (SEM)†	Average prevalence	Average incidence of *B. burgdorferi* infection, 2000–2007	Average LD Incidence, 2008–2011‡	Average yearly change in LD cases, 2008–2011
Nelson	9.55 (1.30)	0.20	0	32.3	+9.7
Appomattox-Buckingham	0.25 (0.11)	0	0.87	4.59	+2.6
Goochland	1.66 (0.33)	0.06	0	0	0
New Kent	1.1 (0.21)	0	0.83	0	0

*LD, Lyme disease.
†Nymphs per 200 m^2.
‡Per 100,000 persons.

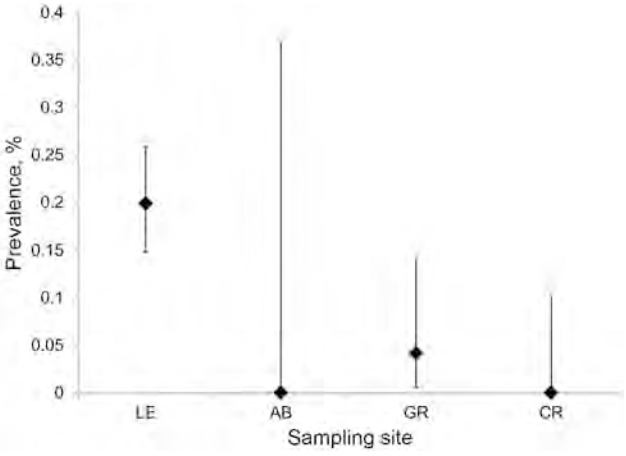

Figure 3. Variation in estimated prevalence of *Borrelia burgdorferi* infection in *Ixodes scapularis* nymphs at 4 field sites in Virginia. Sites are arranged west to east from left to right. LE, Lesesne State Forest; AB, Appomattox-Buckingham State Forest; GR, University of Richmond–owned field site; CR, Crawfords State Forest. Error bars represent 95% CIs.

in Virginia, resulting in increased human exposure to *B. burgdorferi*; the most notable increases in ticks and disease risk are at higher elevations in the western part of Virginia. More generally, our results indicate a dynamic pattern of LD risk. The spatial trends we identified through acarologic sampling are consistent with observed changes in disease incidence and are of paramount public health importance; the observed changes LD epidemiology in Virginia most likely reflect a spatial increase in disease endemicity (Table). We propose that the increase in LD in Virginia is caused by either increasing abundance of *I. scapularis* ticks, increasing prevalence of *B. burgdorferi* infection in the vector, or both. Our data suggest that this vector species may be more abundant than it was before 2007; during widespread collections during 2004–2007, *I. scapularis* ticks existed throughout most of Virginia, and no infected *I. scapularis* ticks were detected in central or western Virginia (*17*,*25*). Similar range expansion of *I. scapularis* ticks has been described in Wisconsin and Michigan (*23*,*24*).

The extent to which the spatial distribution of LD cases in Virginia will continue to change is unclear. Environmental variables previously identified as important drivers of *I. scapularis* abundance may not have uniform effects throughout the range of this species. For example, on the basis of extensive sampling in the eastern United States over several years, Diuk-Wasser et al. estimated an elevational threshold of 510 m for this species (*25*), and Rosen et al. detected more *I. scapularis* on deer at low elevation than high elevation sites in Tennessee (*35*). However, our sampling showed the highest density of host-seeking *I. scapularis* nymphs at elevations approaching this threshold, and we have subsequently collected host-seeking nymphs at >1,000 m in Nelson County in west-central Virginia (R.J. Brinkerhoff, unpub. data). In 2007, a growing focus of LD incidence was observed in southwestern Virginia in Pulaski, Floyd, and Montgomery Counties. These counties have continued to have that region's highest incidence of LD through 2011 (http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm) and mostly occupy high mountain valleys with average elevations of 584–762 m. An elevational threshold that limits tick populations at northern latitudes, where high elevation sites experience extreme cold during winter months, would not be expected where equivalent elevations are associated with more moderate climatic conditions.

Our analysis of LD data from humans indicates that the largest increases in LD incidence since 2007 has occurred in higher-elevation counties in western Virginia; the correspondence between these data and acarologic sampling suggests that the cases reported in these locations most likely are locally acquired and indicate recent spatial and/or numerical expansion of human disease. Our results are notably inconsistent with the findings of surveys of *I. scapularis* ticks on hunter-killed deer in North Carolina and Maryland during 1987–1992, which indicated that *I. scapularis* ticks were most abundant on the Coastal Plain and absent or uncommon in the Appalachian Mountains (*14*–*16*). When human LD surveillance began in Virginia in 1989, the highest incidence was on the state's Eastern Shore (http://www.vdh.virginia.gov/epidemiology/surveillance/surveillancedata/index.htm). This finding was consistent with early surveys of ticks indicating that *I. scapularis* was the most common species in the Coastal Plain and much less common at higher elevations to the west (*14*). A logical conclusion at that time was that LD would continue to spread southward along the state's Coastal Plain. However, during 2000–2011, LD became more prevalent in Virginia's upper Piedmont and Appalachian Mountain zones than in the lower Piedmont and Coastal Plain. The results of older surveys of ticks and recent environmental models are not consistent with the current geographic incidence of LD or our field data. This discrepancy suggests a southwestward spatial expansion of northern tick populations into the upper Piedmont and mountain regions of Virginia or demographic expansion of persons into areas of previously low tick density in western localities. We do not have acarologic data from each county in which LD incidence has increased, nor do we have long-term systematic sampling data, and thus we cannot directly attribute local changes in LD to changes in tick densities.

Analysis of single-nucleotide polymorphisms in the *I. scapularis* genome reinforces the hypothesis that these ticks recolonized northern North America after the most recent glaciation event and that northern populations are

genetically less diverse than southern populations (*21*). Moreover, analyses of single-nucleotide polymorphism data are consistent with south-to-north postglaciation gene flow, whereby northern American-clade populations are a subset of the genetic variation found in southern-clade populations (*21*) resulting from founder effects when ticks recolonized northern latitudes (*22*). Tick populations within both LD-endemic foci show signs of genetic isolation from one another and from southern populations (*22*), and evidence exists for similar lack of gene flow among populations within regions (*19*). Identification of American-clade *I. scapularis* ticks in the southeastern United States (*19*,*33*) might reflect remnant American-clade lineages in the South or might indicate southward dispersal of American-clade ticks. Qiu et al. noted that coastal sites in southern states were associated with strictly American-clade populations, whereas a mix of American- and southern-clade ticks was detected at inland sites (*19*). With respect to our study, we point to the recent lack of detection of *I. scapularis* ticks at high-elevation sites in western or central Virginia (*17*,*25*) and the presence of exclusively American-clade *I. scapularis* ticks in the current study as possible evidence consistent with the population expansion of American-clade ticks from northern population foci. However, we cannot exclude the possibility that the distribution of endemic American-clade ticks simply has expanded in Virginia.

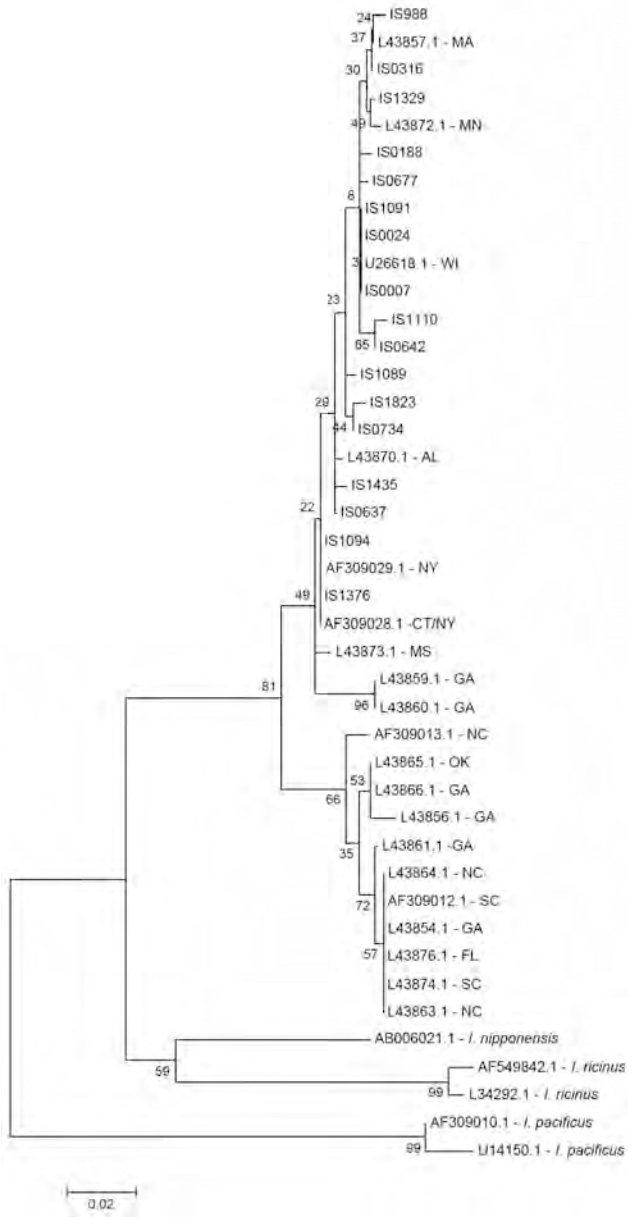

Figure 4. Maximum-likelihood phylogenetic reconstruction of *Ixodes scapularis* lineages based on 16S rRNA gene sequences using Tamura 3-parameter model (*35*). All samples beginning with IS were collected during this study; reference sequence GenBank accession numbers are indicated, as were sampling locations (2-letter state abbreviation). The clade containing samples collected in GA, FL, NC, OK, and SC is known as the Southern clade (sensu Norris et al. [*20*]); the clade containing all samples from this study, indicated by the prefix IS, represents the American clade (more complete explanation of these terms is provided in the text). Bootstrap values at nodes are based on 500 replicates.

Although American- and southern-clade *I. scapularis* ticks are now considered 1 species, apparent differences exist in host-seeking behavior, biting behavior, and duration of attachment to different host types (*9*,*36*,*37*). Genetic differences between the major *I. scapularis* lineages have been well documented (*7*,*19*–*22*), and if American-clade ticks are more likely to feed on humans, the emergence of LD in Virginia would be consistent with increased relative abundance of this variant. In the South, immature *I. scapularis* ticks feed predominantly on low-competence or noncompetent lizard species and are relatively uncommon on rodents (*8*,*36*–*38*). Southern-clade nymphs may have questing behavior that makes them unlikely to be collected on cloth drags or to bite humans (*9*); thus, nymphal ticks are difficult to collect, even in places where adult ticks are common. LD risk should be very low in areas where *I. scapularis* nymphs are unlikely to bite humans, and immature ticks are more likely to feed on reptiles than on competent vertebrate reservoirs. However, data from a single mitochondrial gene, albeit one that has been widely characterized for this species, do not necessarily reflect patterns of differentiation found in nuclear markers (*21*) and probably are not useful for delineating among behavioral phenotypes. Moreover, we sampled in daytime hours during the presumed peak period of nymphal activity (late spring, early summer) and thus would not have detected ticks exhibiting different host-seeking behaviors. It is possible that multilocus genomic analysis or year-round sampling would yield different insights from those reached in this study.

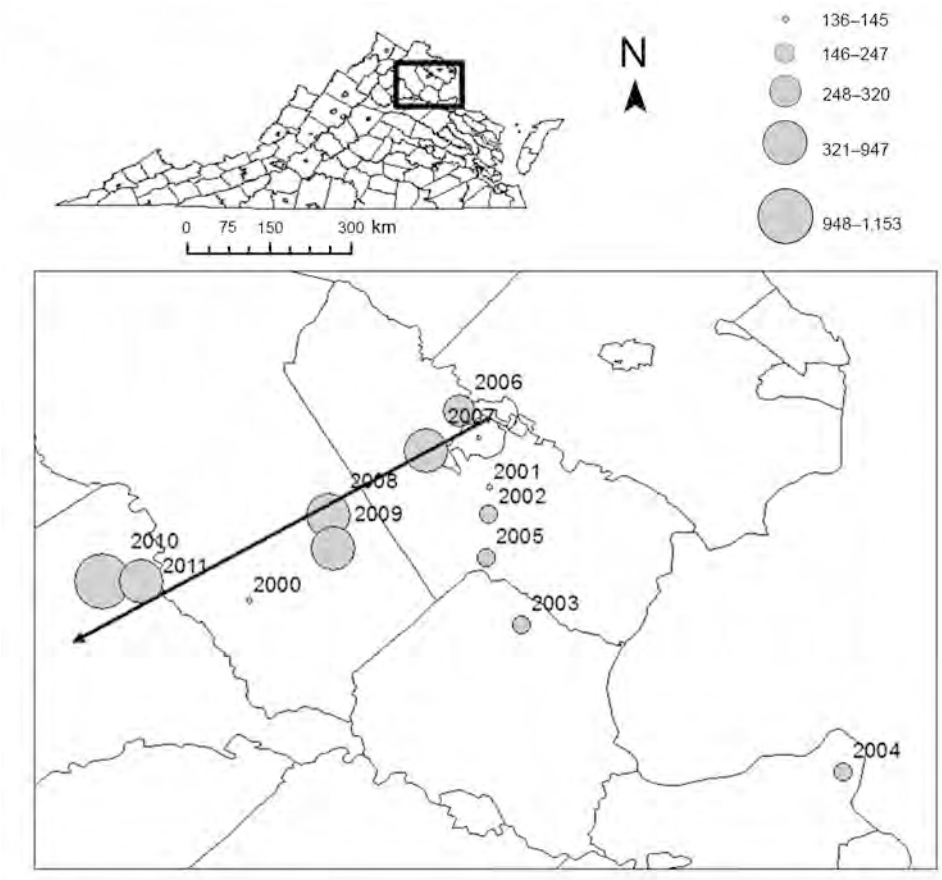

Figure 5. Centroids of annual incidence, by county, Virginia, 2000–2011. The size of each circle represents the annual number of cases reported by the Virginia Department of Health and is proportional to annual incidence (cases/100,000 population). Black arrow represents the mean linear direction of annual movement among centroids during 2006–2011 (these years indicate the recent dramatic increase in Lyme disease incidence in Virginia).

The latitudinal gradient in LD risk in the eastern United States is not easily explained and probably is driven by demographic and environmental factors (*5*,*26*,*39*). However, our data suggest that the boundary between regions to which *I. scapularis* ticks are and are not endemic is moving and that *B. burgdorferi*–infected ticks might be expanding in or into areas from which they historically have been absent. As a result, clinicians and epidemiologists need to be vigilant in the face of changing spatial distributions of risk, especially in transition zones where patterns of disease are rapidly changing (*40*).

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Clinical Isolates of Shiga Toxin 1a–Producing *Shigella flexneri* with an Epidemiological Link to Recent Travel to Hispañiola

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Shiga toxins (Stx) are cytotoxins involved in severe human intestinal disease. These toxins are commonly found in *Shigella dysenteriae* serotype 1 and Shiga-toxin–producing *Escherichia coli*; however, the toxin genes have been found in other *Shigella* species. We identified 26 *Shigella flexneri* serotype 2 strains isolated by public health laboratories in the United States during 2001–2013, which encode the Shiga toxin 1a gene (stx_{1a}). These strains produced and released Stx1a as measured by cytotoxicity and neutralization assays using anti-Stx/Stx1a antiserum. The release of Stx1a into culture supernatants increased ≈100-fold after treatment with mitomycin C, suggesting that stx_{1a} is carried by a bacteriophage. Infectious phage were found in culture supernatants and increased ≈1,000-fold with mitomycin C. Whole-genome sequencing of several isolates and PCR analyses of all strains confirmed that stx_{1a} was carried by a lambdoid bacteriophage. Furthermore, all patients who reported foreign travel had recently been to Hispañiola, suggesting that emergence of these novel strains is associated with that region.

Shiga toxins (Stx) are potent AB_5 cytotoxins that inhibit eukaryotic protein synthesis, eventually leading to host cell death (*1*). Infections with bacteria that produce Stx result in hemorrhagic colitis and can lead to serious complications like hemolytic uremic syndrome (HUS) (*2*). Although 4 species of *Shigella* cause bacillary dysentery, historically only *Shigella dysenteriae* type 1 has been recognized as carrying the genes for Shiga toxin (*stx*). Likewise, *S. dysenteriae* 1 is the only *Shigella* species that causes HUS as a complication of infection (*3*).

The genes encoding the toxin are found in an operon consisting of *stxA* and *stxB*. The *stx* locus in *S. dysenteriae* 1 is surrounded by DNA sequence homologous to lambdoid bacteriophage sequence; however, the toxin genes are not associated with a complete prophage genome (*4*,*5*). Insertion sequences flanking the *stx* region suggest that gene rearrangements occurred and resulted in a defective phage. As a consequence, viable phage are not recovered from *S. dysenteriae* 1 cultures even under conditions that induce phage production (*6*).

Stx has been extensively studied in Shiga toxin–producing *Escherichia coli* (STEC), notably *E. coli* O157:H7. STEC produce 2 variants of Stx: Stx1a (which differs from *S. dysenteriae* 1 Stx by 1 aa), and Stx2 (which shares 56% identity with Stx1a) (*7*,*8*). In contrast to the toxin genes in *S. dysenteriae* 1, those in STEC are generally carried by lambdoid prophages, which integrate into the host bacterial chromosome (*9*). The phage remains in a lysogenic state until environmental conditions induce expression of phage lytic cycle genes, leading to new phage production and lysis of the host bacterium. The stx_{1a} and stx_2 loci are found within the late gene regions of the phage; therefore, induction of the lytic cycle increases expression of the toxin genes and host cell lysis allows for toxin release (*10*).

Recently, acquisition of *stx* genes in clinical isolates of other *Shigella* species has been reported (*11*,*12*). Three cases of infection with *S. dysenteriae* 4 were described, and all were shown to express stx_1. No further characterization of the stx_1-encoding *S. dysenteriae* 4 strains was reported; however, all 3 infected patients had reported recent travel to Hispañiola (*11*). An isolate of Shiga toxin–producing *S. sonnei* from a patient returning from the Ukraine was also characterized; the toxin genes were determined to be

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carried by a lambdoid prophage homologous to *stx*-encoding phages found in STEC (*13*).

We identified 26 clinical isolates of *S. flexneri* 2 that encode stx_{1a}. DNA sequence and PCR analyses determined that stx_{1a} is encoded by a lambdoid prophage. Characterization of the phage indicated that it behaves similarly to *stx*-encoding phages that infect STEC. Like the patients from whom stx_1-encoding *S. dysenteriae* 4 was isolated, patients from whom stx_{1a}-encoding *S. flexneri* 2 was isolated and who reported foreign travel had also recently visited Hispañiola. The potential consequences of an epidemiological link to this region are discussed.

Methods

Bacterial Strains and Growth Conditions

Shigella clinical isolates used in this study are listed in Table 1. *S. flexneri* strains were grown in Tryptic Soy Broth (BD Difco, Franklin Lakes, NJ, USA) at 37°C with aeration or on Tryptic Soy Broth plates containing 1.5% agar and 0.025% Congo red. *E. coli* K-12 strain MG1655 was grown in Luria-Bertani broth and on Luria-Bertani agar plates. Kanamycin and ampicillin were used at 50 μg/mL and 100 μg/mL, respectively.

PCR Analysis of stx_{1a}-Encoding *S. flexneri*

DNA lysates were used for PCR for stx_{1a} with primers stx1-det-F1 and stx1-seq-R1, and for stx_2 with primers stx_F4/stxR1. All strains were verified to contain the virulence plasmid of *S. flexneri* by PCR amplification of *virF* with primers VirF1 and VirF2.

To show that stx_{1a} was phage encoded, we analyzed lysates by PCR with primer pairs Stx1R2/Phage_stxR2 and Phage_stx1F2/Stx1F2. The insertion site of the phage into *S. flexneri* locus S1742 was determined by amplifying the upstream region of S1742 and an early phage gene with primers S1742_up/Stx_phage_up and by amplifying a late phage gene and the downstream region of S1742 with primers Stx_phage_dn/S1742_dn. All PCRs were conducted by using PCR Master Mix (2X) (Fermentas, Pittsburgh, PA, USA) according to the manufacturer's specifications. Primer sequences and expected amplicons are listed in Table 2.

Construction of *recA* Mutants

recA was replaced with a kanamycin-resistance cassette by using λ red recombination (*15*). Primers RecAko-site1 and RecAko-site2 were used to amplify *kan* from pKD4 with 5′ and 3′ overhangs homologous to internal regions of *recA*. Kanamycin-resistant colonies of BS766 (*15*) were double

Table 1. Isolation information for clinical strains of *Shigella flexneri* , USA, 2001–2013*

Strain	Source state (laboratory no.)	Isolation date	Recent travel destination
stx_{1a}-positive			
BS937	HI (HI_N10–094)	2010 Mar	Haiti
BS938	MA (MA_12EN1615)	2012 May	NA
BS942	MA (MA_10EN1901)	2010 Sep	NR
BS943	MA (MA_11EN1036)	2011 Jun	NA
BS951	PA (05E00067)	2005 Jan	NR
BS954	PA (05E02261)	2005 Dec	Dominican Republic
BS955	PA (06E00134)	2006 Jan	NR
BS957	PA (06E00281)	2006 Feb	NR
BS958	PA (06E00283)	2006 Jan	NR
BS959	PA (06E00305)	2006 Feb	NR
BS960	PA (06E00941)	Unknown	Haiti
BS963	PA (08E01943)	2008 Sep	NR
BS965	PA (M09015890001A)	2009 Jul	NA
BS968	PA (M10005231001A)	2010 Feb	Haiti
BS971	PA (M11028960001A)	2011 Nov	Haiti
BS972	MA (12EN7814)	2012 Nov	NR‡
BS974	IN (01–3105)†	2001 Apr	Haiti
BS980	MA (05–3606)†	2005 Oct	NR‡
BS981	CT (06–3001)†	2005 Dec	Dominican Republic
BS982	GA (08–3370)†	2008 Apr	NR
BS988	CT (2012C-3273 †	2012 Jan	Haiti
BS989	CT (2013C-3310)†	2013 (month unknown)	NR
BS998	PA (M13004940001A)	2013 Mar	Haiti
BS999	MA (MA_13EN0428)	2013 Jan	Haiti
BS1010	MD (MDA10005139)	2009 Dec	Dominican Republic
BS1011	MD (MDA12018728)	2012 Jan	NA
stx_{1a}-negative			
BS952	PA (05E00414)	2005 Apr	Peru
BS969	PA (M11015188001A)	2011 Jun	India
BS970	PA (M11015261001A)	2011 Jun	NR

*NA, no information available; NR, none reported; stx_{1a}, Shiga toxin 1a gene.
†Obtained by the Centers for Disease Control and Prevention.
‡No foreign travel reported, but patient had contact with a person who recently returned from Haiti.

Table 2. Primer pairs used for PCR analysis of Shiga toxin–producing *Shigella flexneri*

Primer pair	Sequence, 5′ → 3′	Amplicon size, bp	Source
stx1-det-F1	GTACGGGGATGCAGATAAATCGC	698	(*14*)
stx1-seq-R1	GAAGAAGAGACTGAAGATTCCATCTG		
stx2_F4	GGCACTGTCTGAAACTGCTCCTGT	627	(*14*)
stx2R1	ATTAAACTGCACTTCAGCAAATCC		
VirF1	GCAAATACTTAGCTTGTTGCACAGAG	907	This study
VirF2	GGGCTTGATATTCCGATAAGTC		
VirB01	TTCTACCATCAATCTCCCTTCC	897	This study
IpaAFwd	GTATCTAGCGCCCTCAGCAAG		
IpaHF	GCGTTCCTTGACCGCCTTTCCGATACCG	628	This study
IpaHR	CTTTCAGCCGGTCAGCCACCCTCTGAGAG		
Stx1R2	AGCGAATGACATTCAGCGAATCTA	1,059	This study
Phage_stxR2	GACGCCATACAAGGAGTC		
Stx1F2	ACGCCTGATTGTGTAACTGGAAA	1,333	This study
Phage_stx1F2	CACTCGCGTCACTGTATG		
Stx_phage_up	GACCGCACACTGTGCTATC	1,155	This study
S1742 up	CCGTGCGGGTATTTAACAATAATGG		
Stx_phage_dn	AGTCAAACCGCGCTATTGG	1,224	This study
S1742 dn	TGCATGACAGAGGCAATAAACCCGAT		
RecAko-site1	GCTATCGACGAAAACAAACAGAAAGCGTTGGCGGCAGCACTGGGCCAGA TTGTGTAGGCTGGAGCTGCTTC	1,609	This study
RecAko-site2	AAAATCTTCGTTAGTTTCTGCTACTCCTTCGCTGTCATCTACAGAGAAATCC ATATGAATATCCTCCTTA		
RecA-1	ACATATTGACTATCCGGTATTACCCGG	1,148, 1,701*	This study
RecA-3	GACCGTCCGTGCACACATTATCTATT		

*Amplicon sizes for wild-type *recA* or insertion of *kan* cassette into *recA*, respectively.

purified and screened by PCR with primers RecA-1/RecA-3 for detection of the size difference between chromosomal *recA* and the kanamycin-resistance cassette. This mutant was used as the donor for growing a P1L4 lysate, which was used to transduce the *recA*::*kan* mutation into BS937, BS938, and BS974. Kanamycin-resistant transductants were purified and confirmed by use of PCR, as described above.

Cytotoxicity Assay

The cytotoxicity of bacterial samples for Vero cells was determined as previously described (*16*,*17*). In brief, 100 μL of diluted samples was overlain in 96-well plates containing confluent monolayers of Vero cells and incubated for 48 hours at 37°C in 5% CO_2. Viable cells were fixed with 10% formalin and stained with 0.13% crystal violet. The optical density (OD) of the stained wells was measured at 630 nm by using a BioTek (Winooski, VT, USA) EL800 spectrophotometric plate reader. The CD_{50} (cytotoxic dose that kills 50% of the cells) was calculated by determining the inverse dilution of the bacterial sample that was required to kill 50% of the Vero cells.

In Vitro Neutralization of Stx1a

Overnight supernatants were serially diluted 10-fold in medium. We mixed 100 μL of diluted samples with 100 μL medium, a 1:25 dilution of F45 polyclonal anti-Stx1/Stx1a antiserum (*17*,*18*), or a 1:25 dilution of rabbit polyclonal antiserum against *S. flexneri* whole cell lysate. Toxin samples were incubated with antibody for 2 hours at 37°C in 5% CO_2. We then applied 100 μL of the toxin–antibody mixture to Vero cells and incubated as above.

Mitomycin C Induction of Bacterial Lysis, Shiga Toxin 1a Production, and Prophage Induction

Overnight cultures of bacteria were inoculated 1:100 into Tryptic Soy Broth, and 2 hours after inoculation a final volume of 0.5 μg/mL mitomycin C (Sigma, St. Louis, MO, USA) was added to the cultures. To monitor the induction of bacterial lysis, we read the OD_{600} hourly over a period of 8 hours. Induction of bacterial lysis was noted as a 3-4–fold decrease in OD_{600} compared with the stx_{1a}-negative control strains.

To determine the effect of mitomycin C on production of Stx1a and prophage induction, we collected whole cell lysates and supernatants 3 hours after addition of mitomycin C. Samples were then analyzed for cytotoxicity on Vero cells. For isolation of phage particles, supernatants were prepared similarly, except that a final concentration of 10 mmol/L $MgSO_4$ and a drop of chloroform were added after centrifugation. As described previously, 100 μL of phage lysate was absorbed onto 100 μL of *E. coli* MG1655 for 20 minutes at 37°C (*19*). Molten L-agar top agar (*19*) containing 10 mmol/L $MgSO_4$ was added to the phage/bacteria mixture and poured onto L-agar plates. Plates were incubated overnight at 37°C, and plaque-forming units (PFUs) were counted.

Isolation of Lysogens

Supernatants containing phage were prepared from mitomycin C–induced culture of BS937. Phage lysate was spotted onto an L-soft agar overlay containing either *E. coli* MG1655 or *S. flexneri* 2457T and incubated overnight at 37°C. A loop from the zone of clearing was streaked

for isolation of single colonies, which were subsequently screened for stx_{1a} genes by PCR. Positive colonies from the initial screening were double colony purified, and PCR was repeated to ensure that the colonies were positive for stx_{1a}. MG1655 lysogens were confirmed to not be contaminates of the donor strain, BS937, by testing for *S. flexneri* chromosomal and virulence plasmid genes by use of PCR primer pairs IpaHF/IpaHR and VirB01/IpaFWD (Table 2), respectively. Similarly, 2457T lysogens were analyzed by PCR as described above. Two independently isolated lysogens of MG1655 and 2457T were used for further analysis.

Virulence Assays

Virulence-associated phenotypes were determined by a gentamicin protection invasion assay in HeLa cells and by plaque formation in L2 monolayers, as previously described (*20*,*21*). Both assays were conducted 3 independent times and included technical duplicates or triplicates in each individual experiment.

Whole-Genome Sequencing

DNA was isolated from overnight cultures by using a QIAGEN DNEasy Kit (Valencia, CA, USA). Samples were prepared for sequencing by using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) and sequenced on an Illumina MiSeq sequencing system. The phage sequence was assembled by mapping the reads to the reference phage NC_004913.2 by using Bowtie 2 version 2.1.0 (http://sourceforge.net/projects/bowtie-bio/files/bowtie2/2.1.0/).

Nucleotide Sequence Accession Number

The complete phage sequence of φPOC-J13 from strain BS937 was submitted to GenBank. The sequence is available under accession no. KJ603229.

Results

Identification and Epidemiology of stx_{1a}-positive *S. flexneri* 2 Strains

BS937 and BS938 (Table 1) were acquired from the Hawaii and Massachusetts state laboratories, which had determined the isolates to be positive for stx_{1a} by PCR. Both strains shared the same pulsed-field gel electrophoresis (PFGE) XbaI pattern, JZXX01.0357, as indicated in the Centers for Disease Control and Prevention PulseNet database (http://www.cdc.gov/pulsenet). To identify other clinical isolates of *S. flexneri* 2 that might be stx_{1a}-positive, we searched the PulseNet database for strains that matched this PFGE pattern. From state public health laboratories, we obtained 18 additional strains of *S. flexneri* that matched this PFGE pattern. We also received time-matched, but not PFGE-matched, strains of *S. flexneri* as negative controls. Six additional strains that had been confirmed to be stx_{1a}-positive *S. flexneri* were acquired from the Centers for Disease Control and Prevention. Clinical strains included in this study and their sources are listed in Table 1.

The stx_{1a}-positive *S. flexneri* strains had been isolated over 13 years (2001–2013). They were isolated during all months except August, indicating that seasonality is not involved in the emergence and/or spread of stx_{1a}-encoding *S. flexneri*. Among patients from whom stx_{1a}-positive *S. flexneri* strains were isolated, no incidences of HUS were reported, suggesting that the stx_{1a}-positive *S. flexneri* strains did not cause more severe disease than would typically be caused by such strains lacking stx_{1a}. Among 22 patients for whom travel information was available, 9 reported no foreign travel or knowledge of contact with persons who had traveled. The 13 patients who reported travel had all recently visited Hispañiola or interacted with a traveler who had returned from this region (Table 1).

Verification of Stx1a in *S. flexneri* Isolates

The presence of stx_{1a} in the *S. flexneri* strains was confirmed by PCR (data not shown). All isolates that matched PFGE pattern JZXX01.0357 or had previously been shown to encode stx_{1a} yielded a PCR product of the correct size for the toxin gene. PCR analysis for stx_2 did not produce a product. The 3 negative controls did not generate a PCR product for either stx_{1a} or stx_2.

To determine if Stx1a was released from the bacteria, we tested supernatants from overnight cultures in a Vero cell cytotoxicity assay. All stx_{1a}-positive isolates released a toxin into the supernatant, which killed Vero cells. To confirm that the toxin responsible for killing the cells was Stx1a, we tested overnight supernatants from 3 representative isolates for Vero cell cytotoxicity after neutralization by anti-Stx/Stx1a antiserum. After neutralization, supernatants were no longer cytotoxic to Vero cells (Figure 1). These findings demonstrate that the extracellular product responsible for cytotoxicity to Vero cells is indeed Stx1a and not a different protein being released by the stx_{1a}-expressing *S. flexneri*.

Effects of Mitomycin C on Stx1a and Prophage Production

stx is generally found encoded by functional prophages (*22*). The prophage lytic cycle can be induced with DNA damaging agents (*23*); therefore, to address whether the toxin carried in stx_{1a}-encoding *S. flexneri* was associated with a prophage, we tested sensitivity to lysis when grown in the presence of mitomycin C. All Stx1a-producing *S. flexneri* isolates showed a sharp decrease in OD_{600} within 3–4 hours after addition of mitomycin C, whereas the stx_{1a}-negative strains showed no decrease in OD_{600} (data not shown).

Because all stx_{1a}-encoding *S. flexneri* isolates behaved similarly in the assays, we selected 3 isolates (BS937, BS938, and BS974) to characterize more thoroughly. To further investigate the response to mitomycin C, we grew log-phase cultures and collected samples to measure cell-associated and released toxin. Supernatants and whole cell lysates from bacteria treated with mitomycin C exhibited elevated cytotoxicity to Vero cells (Figure 2). To determine if Stx1a–producing *S. flexneri* generated infectious phage, we analyzed supernatants from untreated and mitomycin C–treated cultures in a PFU assay. After treatment with mitomycin C, PFUs in supernatants of BS937, BS938, and BS974 increased ≈1,000-fold (Figure 3).

Induction of the prophage lytic cycle by mitomycin C is caused by the SOS response (*24*). During the SOS response, the bacterial protease RecA becomes active and cleaves the phage repressor *c*I, which maintains the phage in a quiescent state under non-SOS conditions. Cleavage of *c*I enables transcription of the phage antiterminator Q, which activates the late phage genes, including *stx* (*25*). STEC *recA* mutants no longer release toxin or respond to agents that trigger the SOS response (*24*,*26*,*27*).

To investigate whether similar regulation occurs in Stx1a-producing *S. flexneri*, we constructed *recA* deletions in BS937, BS938, and BS974. *recA* mutants were cultured with mitomycin C as above, and samples were collected to measure the presence of toxin and phage. In the absence of mitomycin C, the *recA* mutants produced and released toxin in amounts comparable to those of the parental strains; however, when cultured with mitomycin C, the *recA* mutants did not exhibit increased cytotoxicity to Vero cells (Figure 2). Additionally, in the absence or presence of mitomycin C, no PFUs were enumerated from the *recA* mutants. Collectively, these data suggest that stx_{1a} in *S. flexneri* is carried by a lambdoid prophage.

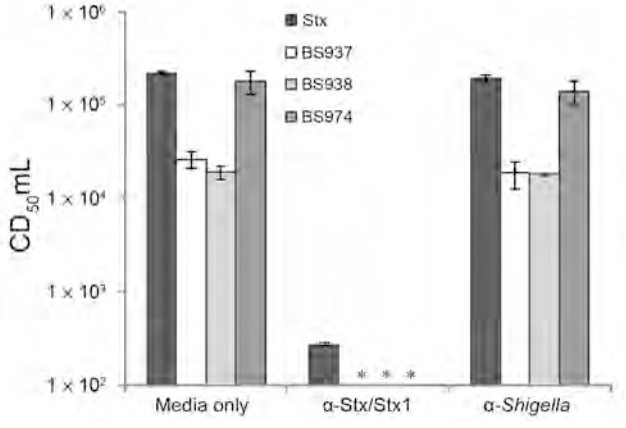

Figure 1. Shiga toxin 1a (Stx1a) is produced and released from stx_{1a}–encoding *Shigella flexneri* isolates. Overnight supernatants from BS937, BS938, and BS974 were serially diluted 10-fold in medium alone, medium containing anti-Stx/Stx1a antiserum, or antiserum against whole cell lysates of *S. flexneri*. After 2 hours of incubation, samples were analyzed in a Vero cell cytotoxicity assay. Stx from *S. dysenteriae* 1 was included as a positive control. CD_{50}/mL is defined as the reciprocal of the dilution of Stx1 that kills 50% of Vero cells. *CD_{50}/mL was below the level of detection. Data are averages of 3 independent experiments. Error bars indicate standard error.

stx_{1a} Carriage by a Lambdoid Prophage in *S. flexneri*

To identify the location of stx_{1a}, we sequenced BS937, BS938, and BS974. Whole-genome sequencing confirmed that stx_{1a} was encoded within a 62-kb lambdoid prophage. To extend the analysis to all the clinical isolates in this study, we used the PCR strategy and primer design shown in Figure 4. To ensure that stx_{1a} in all isolates was phage encoded, we designed primers to amplify from $stxA_{1a}$ and $stxB_{1a}$ (encoding subunits A and B of Stx1a) and 1 kb either upstream or downstream of the stx_{1a} operon. All stx_{1a}-positive strains yielded a PCR product consistent with the toxin being phage encoded. No DNA was amplified from the stx_{1a}-negative isolates.

Whole-genome sequencing revealed that the phage was inserted into locus S1742 (which encodes a putative oxidoreductase) of the *S. flexneri* chromosome. Primers were designed (Figure 4) to determine if the stx_{1a}-encoding phage inserted into S1742 for all isolates. All stx_{1a}-positive strains generated the expected PCR product when amplified with primers specific for the early phage sequence and upstream of S1742 and with primers directed to the late phage sequence and downstream of S1742. None of the stx_{1a}-negative strains yielded an amplified product with the primer pairs. A representative gel of the 4 amplifications for 1 stx_{1a}-negative and 6 stx_{1a}-positive isolates is shown in Figure 5. These data suggest that a phage has integrated into all stx_{1a}-positive isolates. We named this stx_{1a}-encoding phage φPOC-J13.

Lysogeny of Laboratory Strains of *E. coli* and *S. flexneri* with ΦPOC-J13

E. coli MG1655 and *S. flexneri* 2457T were lysogenized as described earlier. To test for production and release of Stx1a, we examined whole cell lysates and supernatants from overnight cultures of MG1655 and 2457T lysogens in Vero cell cytotoxicity assays. The average CD_{50}/mL of supernatants from MG1655 and 2457T lysogens was 1×10^5, similar to that of the stx_{1a}-positive *S. flexneri* clinical isolates. Lysogens were also tested for the presence of phage in supernatants of overnight cultures by determining PFUs. The 2457T lysogens released ≈10^6 PFUs/mL; however, no viable phage could be recovered from MG1655 lysogens.

To confirm that stx_{1a} in the lysogens was phage associated, we conducted PCR amplification as described above (Figure 4). The MG1655 and 2457T lysogens yielded a

PCR product that indicated that the phage regions upstream and downstream of stx_{1a} were present. Lysogens were also tested for integration of the phage into locus S1742 by use of the primers illustrated in Figure 4. In 2457T lysogens, φPOC-J13 inserted into S1742 and produced a PCR product of the expected size. Similarly, in lysogens of MG1655, φPOC-J13 inserted into *ynfG*, the *E. coli* S1742 homologue. These findings demonstrate that the integration of φPOC-J13 is site specific, as has been shown for other lambdoid prophages (*9*).

Virulence Phenotypes of Shiga Toxin 1a–producing *S. flexneri*

We wanted to determine if the presence of φPOC-J13 altered the virulence phenotypes associated with *S. flexneri*. Invasion and plaquing efficiencies of BS937, BS938, and BS974 were compared with those of laboratory strain 2457T (Table 3). BS938 and BS974 exhibited similar invasion efficiency as 2457T; however, invasion with BS937 was significantly higher. All 3 Stx1a–producing *S. flexneri* isolates showed plaquing efficiency comparable to that of 2457T, and the plaque diameters were consistent among all strains. Although it is unclear why BS937 was more invasive, the comparable level of cell-to-cell spread suggests that φPOC-J13 does not appreciably alter the virulence properties of *S. flexneri*.

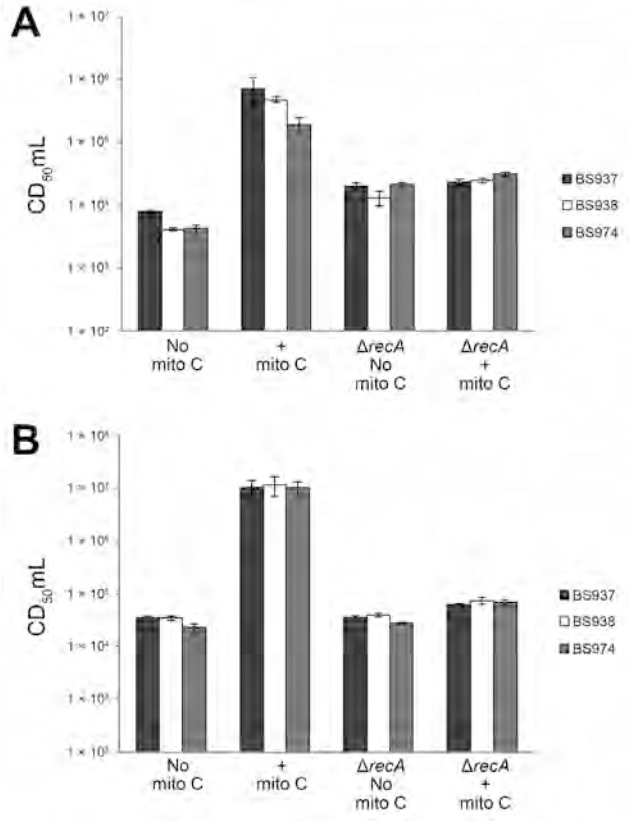

Figure 2. Mitomycin C induces production of Shiga toxin 1a (Stx1a) in a *recA*-dependent manner. Exponentially growing cultures of the indicated parental strains or *recA* mutants were grown with or without 0.5 μg/mL mitomycin C (mito C) for 3 hours. Supernatants (A) or whole cell lysates (B) were prepared for determination of cytotoxicity for Vero cells. CD_{50}/mL values were determined as described in Figure 1. Data are averages of 3 biological replicates. Error bars indicate standard error. Δ indicates samples that are *recA* mutants.

Discussion

Bacteriophages are recognized for their contribution to the genetic diversity of bacteria and for their capacity to transfer virulence factors (*28*). It was first noted in the early 1980s that *stx* in *E. coli* was encoded by a lambdoid bacteriophage (*29*,*30*). We have identified a new stx_{1a}-encoding bacteriophage, φPOC-J13, from clinical isolates of *S. flexneri*. Generally, the acquisition of toxin genes is thought to increase the virulence of a bacterial species. However, according to the available clinical data and our in vitro virulence assays, the production of Stx1a in *S. flexneri* does not seem to increase pathogenicity within the host.

Characterization of φPOC-J13 determined that it behaves similarly to *stx*-encoding phages found in STEC; however, some differences are notable. First, although φPOC-J13 responded to DNA damaging treatment by inducing the lytic cycle and induction was RecA-dependent, *recA* mutants of *S. flexneri* Stx1a–producing strains still maintained a level of Stx1a production and release comparable to that of the noninduced parental strains. In contrast, *recA* mutants of STEC produce a very low level of Stx, and the toxin that is present remains largely cell associated rather than being released into the supernatant (*24*,*26*,*27*). One explanation for the differences in phenotype between Stx1a-producing *S. flexneri recA* mutants and the STEC mutants might be that all STEC *recA* mutants examined encoded stx_2; thus, the regulation of stx_{1a}- and stx_2-encoding phages might vary. Additionally, although Stx1a was produced in *S. flexneri recA* mutants, viable phage particles were not recovered. stx_{1a} has an upstream promoter that is not dependent on induction of the phage lytic cycle (*31*). A similar promoter might be responsible for the baseline level of Stx1a produced in the *S. flexneri recA* mutants and would explain the lack of infectious phage in the mutants.

φPOC-J13 lysogenized laboratory strains of *E. coli* and *S. flexneri*. Viable phage particles were recovered from the supernatants of 2457T lysogens but not from those of MG1655 lysogens, even though Stx1a was produced and released by lysogens of both species. This result might suggest that the stability and/or assembly of φPOC-J13 varies according to the host bacterium. Host differences in the regulation of φPOC-J13 might also account for the discrepancies between the *recA* mutants of Stx1a-producing

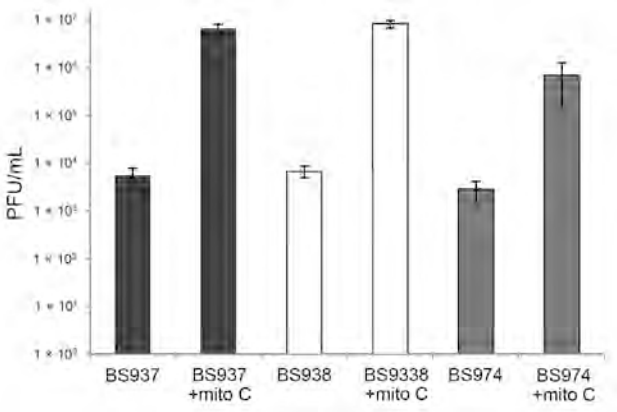

Figure 3. Infectious phage in the supernatants of Shiga toxin 1a gene (*stx*$_{1a}$)–encoding *Shigella flexneri* are induced with mitomycin C (mito C) treatment. Supernatants were collected from exponential cultures of BS937, BS938, and BS974 grown with or without 0.5 μg/mL mito C for 3 h. The number of infectious phage particles was determined by a soft agar overlay method that used *Escherichia coli* MG1655 as the recipient. Plaque forming units (PFUs) of phage lysate were counted after 24 h incubation. Data are averages of 3 independent experiments. Error bars indicate standard error.

S. flexneri and STEC. Our future studies will compare the differences in regulation of *stx*$_{1a}$ in φPOC-J13 with that of known STEC phages.

Another aspect of these *stx*$_{1a}$-encoding *S. flexneri* isolates is their potential epidemiological link to Hispañiola. Although some patients reported no travel, ≈60% reported travel to this region or interaction with a traveler returning from this region. Most of our clinical isolates came from public health laboratories in the eastern United States, suggesting a possible focus in that area. However, the large number of isolates from the eastern United States might simply reflect the large Haitian immigrant population in this region and the resultant frequent travel to Haiti (*32*). Nevertheless, further surveillance of Stx1a-producing *S. flexneri* is warranted to determine the extent of their emergence in Hispañiola.

The epidemiological link to Hispañiola generates many questions about what has led to the emergence of these strains. The earliest Stx1a-producing *S. flexneri* isolates pre-date the earthquake that struck Haiti in January 2010. Thus, this natural disaster is not linked to the presence of *stx*$_{1a}$-encoding *S. flexneri* in the region. One possibility is that the ecosystem in Hispañiola is favorable for the acquisition of φPOC-J13 by *S. flexneri* and possibly other *Shigella* species. An environmental reservoir of *Shigella* spp. has never been identified; therefore, it is tempting to speculate that production of Stx1a might give *S. flexneri* a survival advantage in the aquatic environment. In accordance with this hypothesis, studies on the survival of *Shigella* spp. in amebae indicate that *S. dysenteriae* 1 can persist longer than *S. flexneri* within *Acanthamoeba castellanii* (*33*,*34*). In addition, Stx-producing bacteria can kill the protozoan *Tetrahymena thermophile* to avoid consumption by this predator (*35*). Thus, Stx1a might benefit *S. flexneri* by providing a defense against eukaryotic predators.

It will be important to study clinical isolates of other *Shigella* species and bacterial genera to determine whether they also harbor φPOC-J13; we expect that the occurrence of this *stx*$_{1a}$-encoding phage will be more widespread. Although toxin production in *S. flexneri* did not suggest an increase in pathogenicity, the consequences of the emergence of such Stx1a-producing strains are impossible to

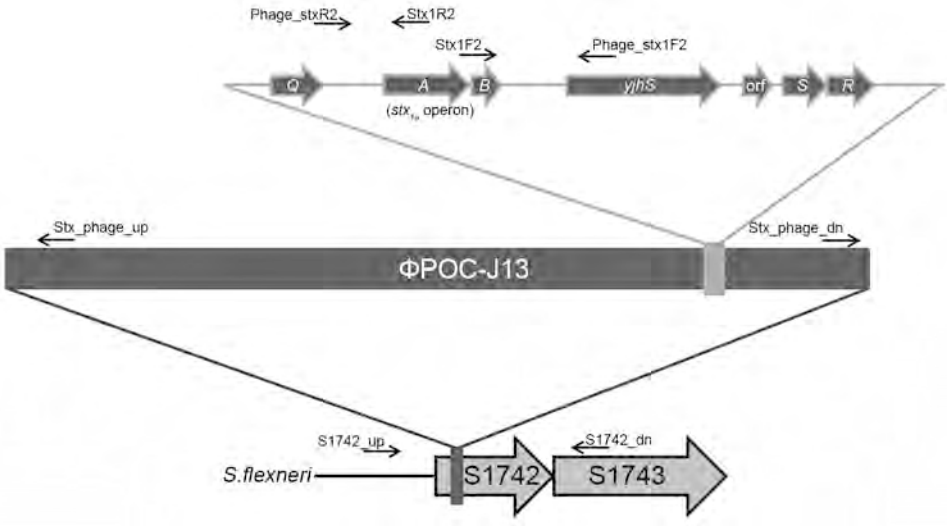

Figure 4. Schematic of PCR designed to determine that Shiga toxin 1a gene (*stx*$_{1a}$) is phage encoded and inserted into the S1742 locus of *Shigella flexneri*. The genetic map shows the insertion of φPOC-J13 into locus S1742 of *stx*$_{1a}$-encoding *S. flexneri*. Location and direction of the primers used for PCR analyses are indicated. The top part of the figure indicates the genes flanking the *stx1a* operon. Q, antiterminator; A and B, Stx1a subunits that form the assembled toxin (A is the *stx1aA* subunit and B is the *stx1aB* subunit); *yshS*, a hypothetical protein that shares homology with *yjhS* from *Escherichia coli* K-12; orf, open reading frame; *S*, gene that encodes the phage holin; *R*, gene that encodes the phage endolysin.

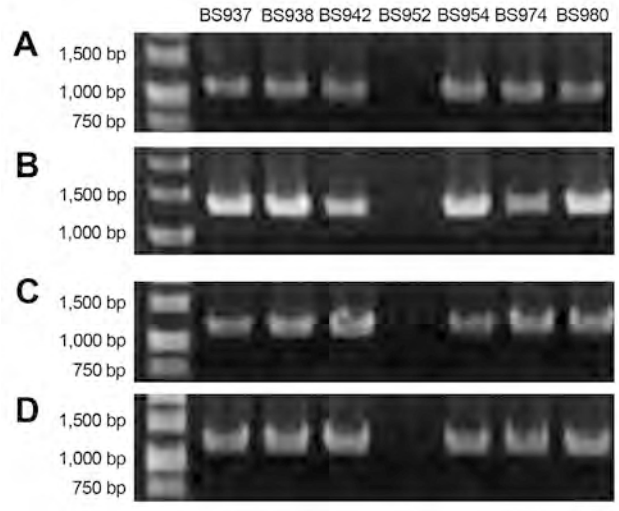

Figure 5. PCR results from representative clinical isolates illustrate that the Shiga toxin 1a gene (stx_{1a}) is phage encoded and inserted into the S1742 locus of *Shigella flexneri*. PCRs based on the primer scheme detailed in Figure 4 are shown for 6 stx_{1a}-positive strains (BS937, BS938, BS942, BS954, BS974, BS980) and 1 stx_{1a}-negative isolate (BS952). To show that stx_{1a} is phage encoded, we used primer pairs Stx1R2/Phage_stxR2 (A) and Phage_stx1F2. Stx1F2 (B). To analyze the insertion of φPOC-J13 into locus S1742, we used primer pairs S1742_up/Stx_phage_up (C) and Stx_phage_dn/S1742_dn (D).

predict. Future studies that address these questions will provide a better understanding of the emergence of stx_{1a}-encoding *S. flexneri*.

Acknowledgments

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Dr Gray is a postdoctoral research fellow at Uniformed Services University of the Health Sciences. Her primary research interests focus on understanding bacterial pathogens that cause intestinal diseases.

Table 3. Virulence properties of *Shigella flexneri* strains

Strain	Invasion, % ± SE*	Plaquing efficiency, % ±SE†
2457T	0.35 ± 0.04	4.34 ± 0.63
BS937	1.24 ± 0.27	3.22 ± 0.56
BS938	0.22 ± 0.03	4.34 ± 0.79
BS974	0.40 ± 0.09	3.24 ± 0.44

*Number of colony-forming units (CFUs) recovered from HeLa cells after gentamicin protection divided by the input CFUs.
†Number of plaques formed on L2 monolayers divided by the input CFUs.

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Prevalence of *Borrelia miyamotoi* in *Ixodes* Ticks in Europe and the United States

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Borrelia miyamotoi, a relapsing fever-related spirochete transmitted by *Ixodes* ticks, has been recently shown to be a human pathogen. To characterize the prevalence of this organism in questing *Ixodes* ticks, we tested 2,754 ticks for a variety of tickborne pathogens by PCR and electrospray-ionization mass spectrometry. Ticks were collected from California, New York, Connecticut, Pennsylvania, and Indiana in the United States and from Germany and the Czech Republic in Europe from 2008 through 2012. In addition, an isolate from Japan was characterized. We found 3 distinct genotypes, 1 for North America, 1 for Europe, and 1 for Japan. We found *B. miyamotoi* infection in ticks in 16 of the 26 sites surveyed, with infection prevalence as high as 15.4%. These results show the widespread distribution of the pathogen, indicating an exposure risk to humans in areas where *Ixodes* ticks reside.

Ixodes ticks can transmit a variety of pathogens, including viruses, bacteria, and protozoa (*1*). *Borrelia* spirochetes are one of the genera of bacteria transmitted by *Ixodes* ticks. Most *Borrelia* that infect ticks belong to the *Borrelia burgdorferi* senso lato group and include *B. burgdorferi* senso stricto, *B. garinii*, and *B. afzelii*, all of which cause Lyme disease in humans (*1*). *Borrelia miyamotoi* has been found in a variety of *Ixodes* ticks and is more closely related to the relapsing fever spirochetes that infect soft ticks than to the bacteria that cause Lyme disease (*2*).

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B. miyamotoi found in Europe and the United States also cause disease in humans (*3*–*5*). A study in Russia has shown that the spirochete *B. miyamotoi* has the ability to infect humans; infections with *B. miyamotoi* cause symptoms similar to those seen with relapsing fever, as well as erythema migrans-like skin lesions on rare occasions (*6*). *B. miyamotoi* has been found in ticks of the following species: *Ixodes scapularis* and *I. pacificus* in the United States, *I. persulcatus* in Japan, and *I. ricinus* and *I. persulcatus* in Europe and Asia (*2*,*7*–*11*). In North America, *B. miyamotoi* has been found as far north as the Canadian provinces of Ontario and Nova Scotia (*12*). In the United States, the geographic range of *B. miyamotoi* is from the Northeast to California and has been reported as far south as Tennessee (*7*,*8*,*13*–*15*). Previous studies have shown that *B. miyamotoi* can be placed into different genetic groups based upon its geographic location and has some variation within the genographic groups (*6*,*9*).

To examine the prevalence distribution and diversity of *B. miyamotoi* in *Ixodes* ticks, we screened individual ticks by PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) to detect tickborne pathogens, including *B. miyamotoi* (*16*). This approach has been used to characterize tickborne microorganisms, including *Ehrlichia* and *Borrelia*, from clinical specimens, heartworms in canine blood, and naturally occurring tick endosymbionts (*16*–*19*). Ticks that tested positive for *B. miyamotoi* were further characterized by using a *Borrelia* genotyping assay to assess genetic diversity (*20*).

Materials and Methods

B. miyamotoi Culture Isolate

The *B. miyamotoi* strain Fr74B was obtained by the Centers for Disease Control and Prevention (Fort Collins, CO, USA), as a culture isolate. This strain was originally isolated from an infected *Apodemus argenteus* field mouse

from Japan. The DNA from this strain was isolated by diluting the culture 1:10 with phosphate-buffered saline and heating to 95°C for 10 min. The raw lysate was then used in the *Borrelia* PCR/ESI-MS genotyping assay (Abbott Laboratories, Des Plaines, IL, USA) at 1 mL per PCR well (*20*).

Ixodes Tick Collection and Extractions

Ticks were obtained from most locations by flagging during 2008–2012. In Germany, a subset of ticks were also obtained after they were removed from persons. The species of *Ixodes* tick was determined by an entomologist and confirmed by the detection of the species-specific endosymbionts (*19*). The numbers and locations of the collection sites are described in Table 1.

Nucleic acids were extracted from ticks according to a published protocol by using bead-beating homogenization followed by isolation of RNA and DNA with DNeasy Blood and Tissue Kit columns (QIAGEN, Valencia, CA, USA) instead of the published QiaAmp Virus Elute Kits (*21*). A negative control consisting of a lysis buffer without a tick was with each set of extractions. Ticks from the United States were processed at Ibis Biosciences (Carlsbad, CA, USA). Ticks collected from the European countries were isolated at their respective sources. Nucleic acid samples from Germany and the Czech Republic were shipped to Ibis at ambient temperatures; those from Czech Republic were shipped after being stabilized by RNAstable (Biomatrica, San Diego, CA, USA) per the manufacturer's instructions.

Molecular Detection and Genotyping of *B. miyamotoi* from Nucleic Acid Extracts

B. miyamotoi was detected and identified by using a previously described broad-range PCR/ESI-MS assay designed to detect tickborne pathogens (*16*). For each set of samples analyzed with the assay, an extraction negative control sample as well as a PCR plate negative-control sample of water was included. A PCR-positive control was already built into the plate for each well in the form of a calibrant (*20*). Amplicons were analyzed by using a research use only PLEX-ID system (Abbott Laboratories). Samples positive for *B. miyamotoi* were further characterized by using a *Borrelia* PCR/ESI-MS genotyping assay as described that is designed to differentiate between *Borrelia* species and genotypes (*20*). PCR/

Table 1. Prevelance of *Borrelia miyamotoi* in *Ixodes* ticks, Europe and the United States, 2008–2012*

Region/subregion	Species	Total no. ticks tested (nymphs; adults)	No. ticks positive for *B. miyamotoi* (% of total)
Czech Republic			
Zavadilka	*I. ricinus*	153 (153; 0)	4 (2.6)
Blatna	*I. ricinus*	100 (100; 0)	2 (2.0)
Dacice	*I. ricinus*	93 (93; 0)	3 (3.2)
Netolice	*I. ricinus*	89 (89; 0)	0 (0)
Germany			
Constance	*I. ricinus*	226 (0; 48)*	4 (1.8)
United States			
Connecticut			
Fairfield County	*I. scapularis*	322 (309; 13)	16 (5.0)
Litchfield County	*I. scapularis*	18 (18; 0)	0
New London County	*I. scapularis*	29 (29; 0)	0
New York			
Dutchess County	*I. scapularis*	357 (357; 0)	2 (0.56)
Suffolk County	*I. scapularis*	180 (24; 156)	2 (1.1)
Westchester County	*I. scapularis*	44 (0; 44)	3 (6.8)
Pennsylvania			
Chester County	*I. scapularis*	80 (79; 1)	2 (2.5)
Indiana			
Pulaski County	*I. scapularis*	81 (0; 81)	10 (12.3)
California			
Alameda County	*I. pacificus*	22 (0; 22)	1 (4.5)
Del Norte County	*I. pacificus*	33 (0; 33)	0
Glenn County	*I. pacificus*	44 (0; 44)	0
Humbolt County	*I. pacificus*	74 (0; 74)	0
Lake County	*I. pacificus*	129 (0; 129)	0
Marin County	*I. pacificus*	85 (0; 85)	1 (1.2)
Mendocino County	*I. pacificus*	57 (0; 57)	2 (3.5)
Napa County	*I. pacificus*	65 (0; 65)	10 (15.4)
Orange County	*I. pacificus*	15 (0; 15)	0
Placer County	*I. pacificus*	250 (0; 250)	4 (1.6)
San Bernardino County	*I. pacificus*	18 (0; 18)	0
Santa Cruz County	*I. pacificus*	64 (0; 64)	0
Sonoma County	*I. pacificus*	126 (126; 0)	2 (1.6)

*A total of 119 ticks were removed from humans, and the life stage of 178 of the 226 ticks tested was not recorded.

ESI-MS assay provides genetic information about the PCR amplicon in the form of A, G, C, and T basecounts, and *B. miyamotoi* detection was defined as positive when one or more primer pairs produced an amplicon basecount signature that was unique to *B. miyamotoi*. Although most researchers agree that the nymphal stage of *Ixodes* ticks is the most epidemiologically essential life stage for transmission of *B. burgdorferi* sensu lato, because little is known about the transmission of *B. miyamotoi* from *Ixodes* ticks to humans, the data for both nymphs and adults were combined.

Sequence Confirmation of *B. miyamotoi* Detections

Representative samples positive for *B. miyamotoi* were selected for 16S Sanger sequencing. Primers were designed to amplify a 676-bp region of the 16S rRNA gene for *Borrelia*. A M13 tag was added to each primer for sequencing. The M13 forward sequence tag was 5′-CCC AGT CAC GAC GTT GTA AAA CG-3′, and the reverse tag was 5′-AGC GGA TAA CAA TTT CAC ACA GG-3′. The forward primer used was 5′-M13-CGC TGG CAG TGC GTC TTA AG-3′, and the reverse primer was 5′-M13-GCG TCA GTC TTG ACC CAG AAG TTC-3′. The amplification of the 16S rRNA genes was performed in a 50 mL reaction containing 1 mL nucleic acid extract, 1 unit of Platinum Taq High Fidelity polymerase (Invitrogen, Carlsbad, CA, USA) or Immolase Taq (Bioline, Randolph, MA, USA), the manufacturer's PCR buffer, 2.0 mmol/L $MgSO_4$, 200 μmol/L dATP, 200 μmol/L dCTP, 200 μmol/L dTTP, 200 μmol/L dGTP (Bioline), and 250 nmol/L of each primer. The following PCR cycling conditions were used on an MJ Dyad 96-well thermocycler (Bio-Rad Inc., Hercules, CA, USA): 95°C for 2 min, followed by 8 cycles of 95°C for 15 s, 50°C for 45 s, and 68°C for 90 s, with the 50°C annealing temperature increasing 0.6°C for each cycle. PCR was continued for 37 additional cycles of 95°C for 15 s, 60°C for 15 s, and 68°C for 60 s. The PCR cycle ended with a final extension of 4 min at 72°C. Reactions were visualized by electrophoresis on 1% agarose gels to ensure the presence of appropriately-sized products before being sent to SeqWright (Houston, TX, USA) for purification and sequencing with M13 primers. Resulting sequences were trimmed of primer sequences and a consensus created. The consensus sequence was analyzed with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the nucleotide database to determine the species.

Results

Multilocus PCR/ESI-MS Genotyping of *B. miyamotoi*

The multilocus *Borrelia* PCR/ESI-MS genotyping assay differentiates strains and species of *Borrelia* by their unique combination of basecount signatures. To characterize the prevalence of *B. miyamotoi* in *Ixodes* ticks we examined the basecount signatures from ticks that were positive for *B. miyamotoi*. Positive specimens from each of the 3 regions (United States, Europe, and Japan) typically produced basecount signatures at 5 of the 8 loci evaluated in the *Borrelia* genotyping assay. Based upon these 5 signatures, *B. miyamotoi* from the United States, Europe, and Japan are distinct genotypes (Table 2). All the specimens from North America had the same basecount signatures for the 5 detecting primer pairs. A separate signature combination was found for all of the European isolates detected in ticks from Germany and the Czech Republic. A third signature was observed from the CDC culture isolate from the Japanese strain. Although all 3 genotypes shared the same basecount for the locus BCT3515, the European genotype did not have any other basecount signatures in common with the other 2 genotypes. The North American and Japanese genotypes had the same signatures for 2 of the 4 remaining loci, BCT 3519 and BCT3511. We detected *B. miyamotoi* with 3 or more primers in the *Borrelia* genotyping assay in all but 4 of the 68 positive specimens. Several factors may explain why all 5 primers did not detect the bacteria, including nucleic acid quality and quantity or differences in primer sensitivities.

Prevalence of *B. miyamotoi* in Europe and the United States

I. ricinus ticks from the Czech Republic and Germany in Europe and *I. scapularis* and *I. pacificus* ticks from 5 states in the United States were screened for *B. miyamotoi* by PCR/ESI-MS. *B. miyamotoi* was found in all regions examined in varying degrees (Table 1) and in all 3 *Ixodes* species examined. Germany had a low incidence rate; only 4 of the 226 ticks tested were infected (1.8%). Incidence of *B. miyamotoi* infection of ticks from the Czech Republic varied by region and ranged from 0% to 3.2% with an average infection rate of 2%. In North America, the infection rates of ticks varied from 0% to 15.4%. All negative controls were negative and all positive controls were positive.

Table 2. *Borrelia miyamotoi* PCR/ESI-MS basecount signatures*

Region	Genotype	BCT3515 (*rplB*)	BCT3517 (*flaB*)	BCT3519 (*hbb*)	BCT3520 (*hbb*)	BCT3511 (*gyrB*)
Europe	1	A13G22C15T18	A41G30C23T27	A41G29C19T46	A52G29C13T47	A36G32C13T35
North America	2	A13G22C15T18	A43G28C23T27	A40G30C18T47	A52G30C13T46	A37G31C13T35
Japan	3	A13G22C15T18	A41G29C23T28	A40G30C18T47	A53G29C13T46	A37G31C13T35

*PCR/ESI-MS, PCR and electrospray ionization mass spectrometry.

Sequence Confirmation of *B. miyamotoi* detections

Representative samples were selected for 16S rRNA sequencing: 1 sample from Pennsylvania in the United States, 1 from Germany, and 1 from the Czech Republic. The samples from Germany and the Czech Republic were identical (KF740842 and KF740841, respectively) and matched 99.11% (669 bp out of 675 bp) of the *B. miyamotoi* LB-2001 sequence, a North American isolate from the East Coast (GenBank accession no. NC_022079). The sample from Pennsylvania (KF740843) was identical (675 bp of 675 bp) to the *B. miyamotoi* LB-2001 sequence.

Discussion

In this study, we identified 3 distinct *B. miyamotoi* genotypes in the United States, Europe, and Japan. Results show that *B. miyamotoi* is widely distributed across North America and Europe.We observed no genotypic differences using this PCR/ESI-MS assay between the *B. miyamotoi* detected in *I. scapularis* from the eastern US states and the midwest or between these bacteria and the *B. miyamotoi* detected in *I. pacificus* from California. In a study by Mun et al., a 766-bp region of the flagellin gene sequence were shown to have and a 0.9% difference between *B. miyamotoi* found in *I. pacificus* and those found in *I. scapularis* in the United States (*8*). However, our flagellin primers targeted a region of the flagellin gene that does not contain the differences identified by Mun et al., thus explaining why we found a single North American genotype. Previous studies that examined the sequence of the 16S rRNA gene from multiple *B. miyamotoi* strains indicated that strains from the United States and Europe were located in their own clusters (*6*). The Japanese strain FR64b grouped with isolates found in infected humans and *I. persulcatus* ticks in Russia, whereas the *B. miyamotoi* found in *I. ricinus* ticks from Russia grouped with those found in Europe (*6*). In our genetic analysis, the Japanese strain also differed from that found in *I. ricinus* in Europe.

Our study demonstrates that the presence of *B. miyamotoi* in *Ixodes* ticks is widespread across the regions examined and was observed in all 3 species of field-collected *Ixodes* ticks. In Europe we observed *B. miyamotoi* in ≈2.0% of *I. ricinus* ticks tested, consistent with the detection rates in other studies examining *I. ricinus* prevalence at other locations in Europe (*9*,*10*). Our detection rates were also similar to those seen in an earlier study on ticks from Mendocino County, California (*8*). *I. scapularis* ticks from the East Coast region (New York, Connecticut, and Pennsylvania) were found to have infection rates ranging from 0% to 6.8% for ticks. In Indiana, however, a much higher percentage, ≈12%, of *I. scapularis* ticks examined were infected with *B. miyamotoi*. Other studies have also shown that local site-to-site prevalence of *B. miyamotoi* can vary greatly from the overall regional mean (*13*).

Our study indicates that *B. miyamotoi* is likely present in any region where *Ixodes* ticks reside but that infection rates can vary greatly by region. Since the original description of *B. miyamotoi* as a human pathogen, studies have shown clinical infection in both healthy and immunocompromised patients in both Europe and the United States (*3*–*6*,*22*). If physicians know the regional infection rate in ticks, they will be alert for possible exposure risks for their patients. Standard Lyme borreliosis serologic tests offered by commercial laboratories cannot be relied on to detect *B. miyamotoi* infection in patients. *B. miyamotoi* has been shown to have transovarial transmission, suggesting that larval ticks may also pose a risk (*7*). Little is yet known about the transmission rates to humans, and further studies are required to better gauge the risk to humans in these *B. miyamotoi*-endemic regions.

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Dr Crowder is a researcher at Ibis Biosciences working on vectorborne disease diagnostics. His research interests include tick-transmitted diseases in both the vector and in clinical patients.

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Biomarker Correlates of Survival in Pediatric Patients with Ebola Virus Disease

Anita K. McElroy, Bobbie R. Erickson, Timothy D. Flietstra, Pierre E. Rollin, Stuart T. Nichol, Jonathan S. Towner, and Christina F. Spiropoulou

Outbreaks of Ebola virus disease (EVD) occur sporadically in Africa and are associated with high case-fatality rates. Historically, children have been less affected than adults. The 2000–2001 Sudan virus–associated EVD outbreak in the Gulu district of Uganda resulted in 55 pediatric and 161 adult laboratory-confirmed cases. We used a series of multiplex assays to measure the concentrations of 55 serum analytes in specimens from patients from that outbreak to identify biomarkers specific to pediatric disease. Pediatric patients who survived had higher levels of the chemokine regulated on activation, normal T-cell expressed and secreted marker and lower levels of plasminogen activator inhibitor 1, soluble intracellular adhesion molecule, and soluble vascular cell adhesion molecule than did pediatric patients who died. Adult patients had similar levels of these analytes regardless of outcome. Our findings suggest that children with EVD may benefit from different treatment regimens than those for adults.

Outbreaks of Ebola virus disease (EVD) occur sporadically in sub-Saharan Africa and are associated with exceptionally high case-fatality rates (CFRs). The disease onset is nonspecific and is characterized by abrupt onset of fever, fatigue, headache, myalgia, and gastrointestinal distress 3–13 days after exposure to the virus (*1*). The term hemorrhagic fever has been used to describe this disease process because hemorrhagic manifestations develop in many patients during the course of illness. The *Ebolavirus* genus includes 5 different viruses that result in different CFRs: Ebola virus (EBOV; CFR 57%–90%), Sudan virus (SUDV; CFR 41%–65%), and Bundibugyo virus (CFR 40%) cause fatal infections, but neither Tai Forest virus nor Reston virus has been associated with human fatalities (*2*,*3*).

Pediatric patients have been underrepresented in EVD studies because total numbers of affected children in any given EVD outbreak, whether associated with EBOV, SUDV, or Bundibugyo virus, are usually low because of outbreak dynamics and societal structure. For example, nosocomial EVD infections mostly occur in adults working on hospital wards, and children are not usually caregivers for EVD patients. However, the 2000–2001 SUDV outbreak in the Gulu district of Uganda, the largest recorded EVD outbreak to that point, resulted in 425 cases; 145 cases were in patients ≤21 years of age, and 55 of these cases were laboratory confirmed (*4*,*5*). The CFR for pediatric patients in this outbreak was lower than for adults (*6*), but the reasons for this increased survival were unknown. The relatively large number of pediatric cases in this outbreak enabled closer investigation of factors associated with increased survival of pediatric patients with EVD.

Samples collected during the Gulu outbreak have been invaluable for advancing understanding of EVD pathophysiology. Studies using these samples found associations between fatal outcomes and elevated liver enzyme levels, renal dysfunction, cytokine dysregulation, and genetic factors (*7*–*9*). Recently, we analyzed serum biomarkers by using samples from the Gulu outbreak and identified associations between cytokines/chemokines, acute-phase reactants, makers of coagulopathy, and markers of endothelial function and patient death, hemorrhage, and viremia (*10*). In this study, we used a series of multiplex assays to measure the concentrations of 55 serum analytes in specimens from patients from the Gulu outbreak to identify biomarkers that had age-specific associations with survival, hemorrhagic manifestations, or both.

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Materials and Methods

Study Design

During the 2000–2001 Gulu EVD outbreak, an international response team, including representatives from the US Centers for Disease Control and Prevention (CDC), provided clinical and technical assistance. Serum samples were obtained as part of the management of these patients and were stored in liquid nitrogen. In addition to the samples from the 55 pediatric patients (≤21 years of age) who had laboratory-confirmed EVD, we selected samples from 50 adult patients (>21 years of age) who had laboratory-confirmed infection; this selection was designed to be representative of overall sex ratios, hemorrhagic manifestations, and death rates observed during the outbreak. A total of 45 of the 55 pediatric patients (70 specimens total) and 49 of the 50 selected adult patients (127 specimens total) had sufficient serum available for the proposed studies. Data on all patients used in this study, including age, sex, illness outcome, presence of hemorrhagic manifestations, and number of specimens available, are shown in Table 1 (http://wwwnc.cdc.gov/EID/article/20/10/14-0430-T1.htm).

Specimens were prioritized for novel analyses first; if a sufficient sample amount was available, serum chemistry analyses were also performed. All samples were inactivated by γ-irradiation (5×10^6 rad) before use, as previously described (*11*). Institutional Review Board approval was obtained before the study was initiated, and an exemption was granted by the CDC Human Research Protection Office.

Bead-based Multiplex Assays

The following assays were purchased from Affymetrix (Santa Clara, CA, USA) and performed according to the manufacturer's instructions: a 26-plex assay for granulocyte-macrophage colony-stimulating factor, growth-regulated oncogene α, interferon (IFN) α2, IFNβ, IFNγ, IFNγ-inducible protein 10 (IP-10), interleukin 10 (IL-10), IL-12 (p70), IL-12 (p40), IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-1 receptor antagonist, monocyte chemoattractant protein 1, macrophage colony-stimulating factor, macrophage inflammatory protein 1α and 1β, soluble CD40 ligand, soluble E-selectin, soluble Fas ligand, tumor necrosis factor α, and vascular endothelial growth factor A; a 2-plex assay for D-dimer and tissue plasminogen activator; a 5-plex assay for plasminogen activator inhibitor 1 (PAI-1), serum amyloid antigen (SAA), regulated on activation, normal T-cell expressed and secreted (RANTES), soluble intracellular adhesion molecule (sICAM) 1, and soluble vascular cell adhesion molecule (sVCAM) 1; and single-plex assays for C-reactive protein and fibrinogen. Single-plex assays for ferritin and cortisol and 2-plex assays for tissue factor (TF) and thrombomodulin were performed according to the manufacturer's instructions (Millipore, Billerica, MA, USA). For samples with values outside the upper end of the standard curve, additional dilutions were made as necessary to obtain accurate values for all analytes.

ELISAs

Mannose-binding lectin (Hycult Biotech, Plymouth Meeting, PA, USA) and total IgG (eBioscience, San Diego, CA, USA) ELISAs were performed according to the manufacturers' instructions. For samples with values outside of the upper range of the standard curve, additional dilutions were made as necessary to obtain accurate values for all analytes.

Serum Chemistry Testing

A total of 135 of the 197 patient samples had enough volume for serum chemistry analyses. A Piccolo comprehensive metabolic reagent disk was run on the Piccolo xpress Chemistry Analyzer (Abaxis, Union City, CA, USA) to determine serum chemistry values for alanine aminotransferase, albumin, aspartate aminotransferase, alkaline phosphatase, calcium, chloride, creatinine, glucose, potassium, sodium, total bilirubin, total carbon dioxide, total protein, and blood urea nitrogen.

Viremia Analysis

Total RNA was purified from serum samples by using the MagMAX-96 Viral RNA Isolation Kit on the MagMAX Express-96 Magnetic Particle Processor (Ambion, Grand Island, NY, USA). In parallel, RNA was purified from serial dilutions of serum samples from healthy persons that were inoculated with a known titer of SUDV and used to generate a standard curve. Real-time PCR was then performed as previously described (*12*).

Statistical Analysis

An analysis of variance (ANOVA) was conducted for each of the 55 analytes by using patient sex, patient age, days after symptom onset, viremia, outcome, hemorrhage, and HIV status as independent variables. To represent the normal course of illness, only samples taken 0–15 days after symptom onset were analyzed. All variables were converted to categorical variables because of the number of samples and the distribution of the numerical data. An initial overall α of 0.25 was set, and the Bonferroni inequality was used to arrive at a critical p value of 0.0045 for the individual analytes. Because multiple testing affects the statistical power of the tests, the technique of Yoav and Hochbergs (*13*) was used to control the false discovery rate to balance the power and type 1 error of the analyses.

Using these criteria, 10 analytes showed significant results for age (Table 2). The independent variables were

Table 2. Biomarkers that demonstrated an association with age in analysis of Ebola virus disease, Uganda, 2000–2001*

Biomarker	p value
sVCAM	1.72×10^{-22}
sICAM	3.23×10^{-22}
SAA	5.42×10^{-22}
PAI-1	5.77×10^{-16}
RANTES	1.97×10^{-10}
MCSF	1.87×10^{-6}
Total IgG	3.35×10^{-6}
IP-10	2.34×10^{-5}
Tissue factor	1.79×10^{-3}
Interleukin 10	3.20×10^{-2}

*p values determined by analysis of variance. IP-10, interferon γ–inducible protein 10; MCSF, macrophage colony-stimulating factor; PAI-1, plasminogen activator inhibitor-1; RANTES, regulated on activation, normal T cell expressed and secreted; SAA, serum amyloid antigen; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intracellular adhesion molecule.

analyzed to measure co-linearity, but no significant variance inflation was observed. After ANOVA, stepwise regression for each significant analyte was used to find the best model, excluding nonsignificant variables ($p \geq 0.05$). Because a primary focus of this study was the relationship between age and survival in association with these analytes, we added an age/death interaction term for those models where results for age and/or death were significant. This interaction term was significant for 6 analytes (Figure 1). Viremia and death interactions were also examined, but no analytes showed a significant interaction. Age and hemorrhage interactions showed significant results for 2 analytes (Figure 2). We compiled the analytes that were significantly different for these variables and calculated the means and SEs for each time interval. The distribution of the viremia levels was clearly not normal, so the Mann-Whitney U test was used to determine differences in viremia status for adult versus pediatric patients and those with fatal versus nonfatal outcomes within each age group. Patients with no measurable viremia levels were excluded from analysis. A more detailed description of the statistical methods we used is found in our previous study (*10*).

Results

Patient Characteristics

Patient characteristics for the final study population are shown in Table 3. CFRs for the 55 laboratory-confirmed pediatric cases varied by age: 76.9% for children ≤5 years of age (n = 13), 37.5% for children 6–15 years of age (n =16), and 41.6% for adolescents 16–21 years of age (n = 26). In contrast, the CFR for the 161 adults with laboratory-confirmed cases was 56.5%. For the 45 pediatric patients for whom serum samples were available for analysis, the CFR was 100% for children ≤5 years of age (n = 8), 28.6% for those 6–15 years (n = 14), and 39% for those 16–21 years

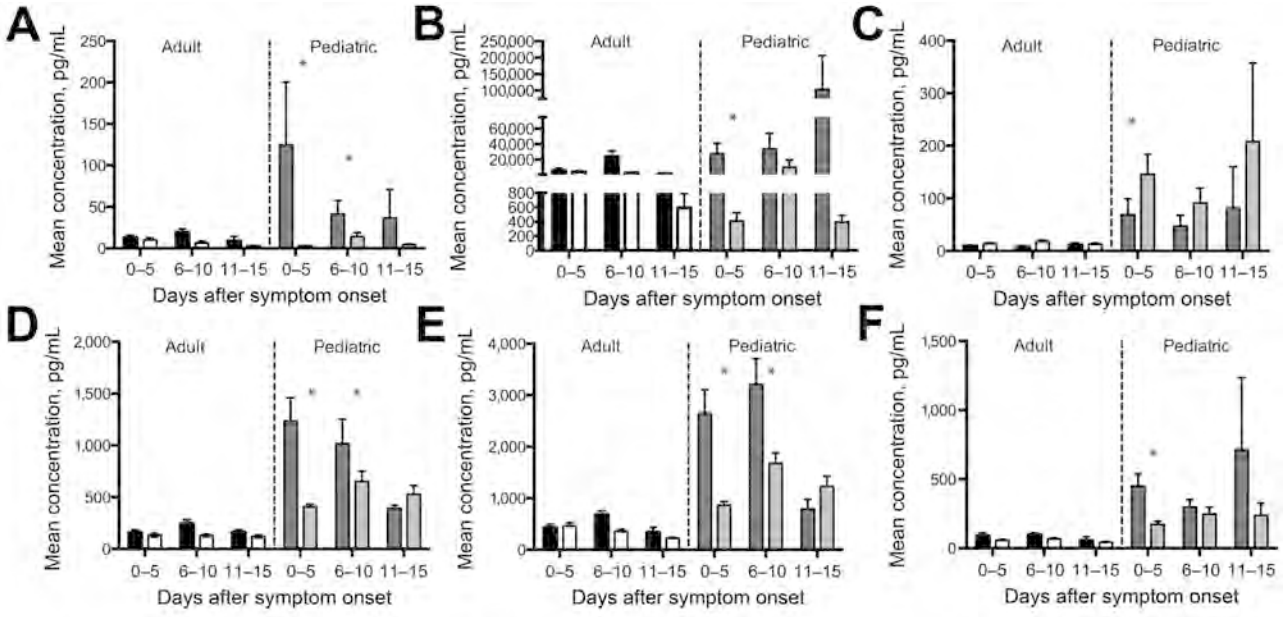

Figure 1. Biomarkers associated with age-dependent survival outcomes for patients with Ebola virus disease: black, adult fatal; white, adult nonfatal; dark gray, pediatric fatal; light gray, pediatric nonfatal. A) Interleukin 10; B) interferon g–inducible protein 10; C) regulated on activation, normal T cell expressed and secreted; D) soluble intracellular adhesion molecule; E) soluble vascular cell adhesion molecule; F) plasminogen activator inhibitor 1. Mean levels are depicted in each patient group as a function of time after symptom onset. Error bars represent SE; * indicates $p \leq 0.05$. Numbers of specimens included in each group are as follows: adult fatal at 0–5 days, 27; adult nonfatal at 0–5 days, 20; adult fatal at 6–10 days, 22; adult nonfatal at 6–10 days, 24; adult fatal at 11–15 days, 5; adult nonfatal at 11–15 days, 14; pediatric fatal at 0–5 days, 10; pediatric nonfatal at 0–5 days, 15; pediatric fatal at 6–10 days, 5; pediatric nonfatal at 6–10 days, 13; pediatric fatal at 11–15 days, 2; pediatric nonfatal at 11–15 days, 4.

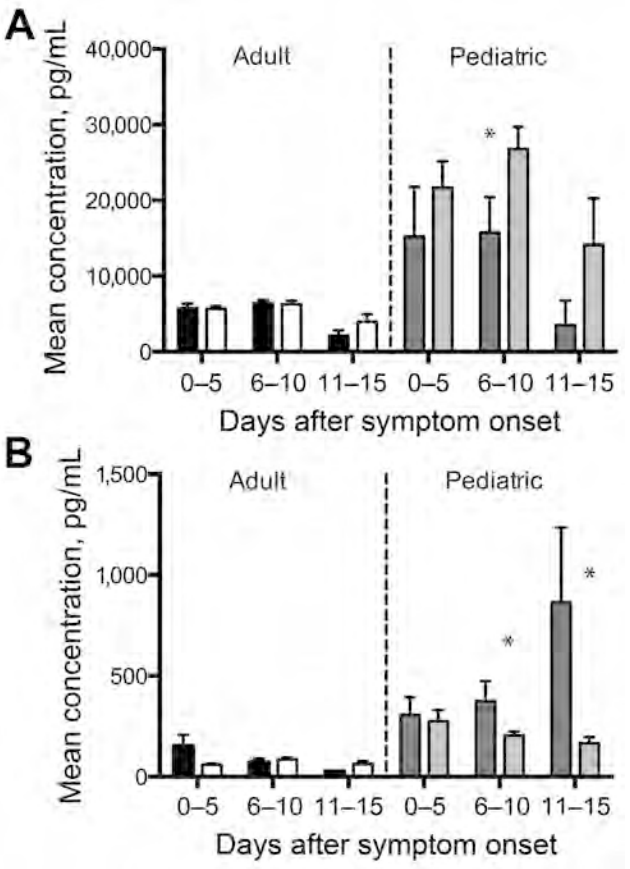

Figure 2. Biomarkers associated with age-dependent hemorrhagic manifestations (heme) for patients with Ebola virus disease: black, adult heme; white, adult nonheme; dark gray, pediatric heme; light gray, pediatric nonheme. A) Serum amyloid antigen; B) plasminogen activator inhibitor 1. Mean levels are depicted in each patient group as a function of time after symptom onset. Error bars represent SE; * indicates p≤0.05. Numbers of specimens included in each group are as follows: adult heme at 0–5 days, 9; adult non-heme at 0–5 days, 38; adult heme at 6–10 days, 12; adult non-heme at 6–10 days, 34; adult heme at 11–15 days, 9; adult non-heme at 11–15 days, 10; pediatric heme at 0–5 days, 7; pediatric non-heme at 0–5 days, 18; pediatric heme at 6–10 days, 6; pediatric non-heme at 6–10 days, 12; pediatric heme at 11–15 days, 2; pediatric non-heme at 11–15 days, 4.

(n =23). Because all samples were available only from fatal cases for children ≤5 years of age, we initially excluded these samples from biomarker statistical analyses. However, because this exclusion might have introduced bias, we subsequently repeated the analysis including these 9 additional specimens and found no differences in the statistical significance of the models. Because the CFRs for child and adolescent populations were lower than those for adults, the 3 pediatric age groups were combined to increase the power of the study.

Patients were considered to have hemorrhagic manifestations if they exhibited any of the following signs: vomiting blood; blood in the stool; or bleeding from the gums, skin, or eyes. We found that a higher percentage of pediatric than adult patients exhibited hemorrhage, but overall CFR remained lower for children than for adults.

Viremia

To determine whether pediatric patients were more likely to survive as a result of lower levels of viral replication, we measured viremia levels in each sample by using real-time reverse transcription PCR and compared the results with a standard curve generated from stock virus of known titer. No statistically significant differences were found between viral loads in adults and pediatric patients (Figure 3, panel A). Viral loads were higher for patients who died (Figure 3, panel B), as previously demonstrated (*12*); however, in the pediatric population, this difference did not reach statistical significance, likely because of the small sample size and the wide range of observed values in the pediatric patients with nonfatal cases.

Serum Chemistry Testing

Serum chemistry tests were performed on all samples that had sufficient available volume after initial testing. Blood urea nitrogen, creatinine, and albumin levels varied by age, as expected, given the normal physiological differences between adults and children (data not shown). No age-specific associations were found between any analyte in the serum chemistry results and death or hemorrhage. More labile analytes, such as carbon dioxide and electrolytes, were excluded from analysis.

Biomarkers of Inflammation

Cytokines and chemokines are a diverse group of proteins that modulate the immune response and have been extensively studied in many different disease processes. We analyzed 25 cytokines and chemokines. Of the 10 analytes that had a statistically significant association with age (Table 2), 4 were cytokines or chemokines, and 3 of those—IL-10, IP-10, and RANTES—were associated with an age-dependent survival outcome (Figure 1, panels A–C). IL-10 and IP-10 levels were higher in pediatric patients who died than in those who survived; adult patients had similar levels of these biomarkers regardless of outcome (Figure

Table 3. Characteristics of patients with laboratory-confirmed Ebola virus disease during outbreak in Uganda, 2000–2001*

Characteristic	No. (%) patients	
	Pediatric, n = 37	Adult, n = 49
Sex		
F	23 (62.2)	33 (67.3)
M	14 (37.8)	16 (32.7)
Hemorrhage	15 (40.5)	16 (32.7)
Fatal outcome	14 (37.8)	27 (55.1)

*Pediatric patients were 6–21 y of age, adult patients 22–60 y of age. Eight patients ≤5 years of age were excluded from this portion of the analysis (see text).

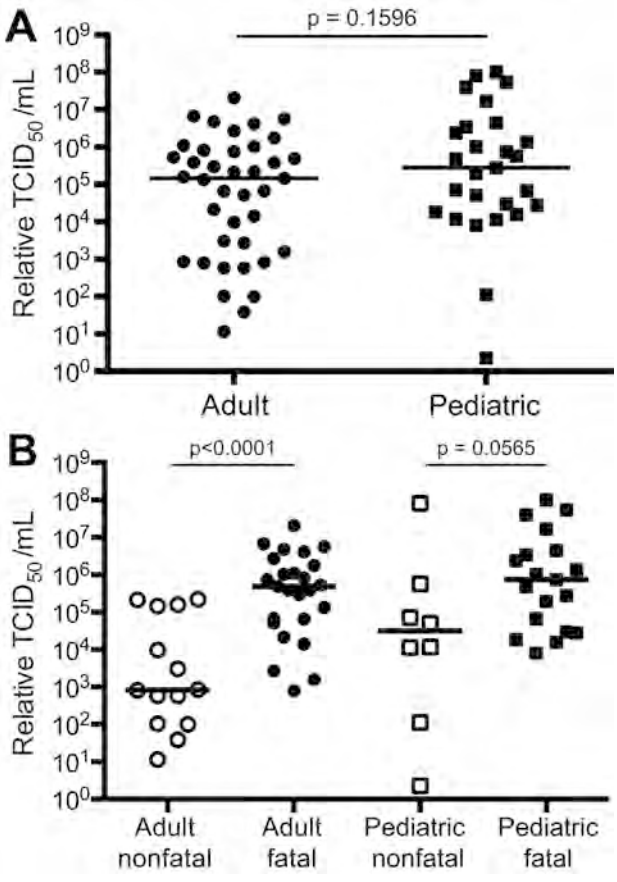

Figure 3. Viral loads for Ebola virus disease patients infected with Sudan virus during outbreak in Uganda, 2000–2001. A) Relative $TCID_{50}$, of pediatric patients (1–21 years of age) compared with those of adult patients (22–60 years of age); B) fatal and nonfatal outcomes for pediatric versus adult patients. Viral load determination was performed on all samples and quantitated by a reverse transcription PCR curve generated from a known titer stock of Sudan virus. Error bars represent SE. $TCID_{50}$, 50% tissue culture infective dose.

1, panels A, B). Serum samples from this outbreak have been analyzed in the past, and increased levels of IL-10 were reported in patients with fatal outcomes (*8*); however, age was not analyzed in the prior study. RANTES levels were higher in pediatric patients than in adult patients and were further elevated in pediatric patients with nonfatal outcomes (Figure 1, panel C). RANTES is the only biomarker we identified as associated with increased survival in pediatric patients. Macrophage colony-stimulating factor levels were higher in pediatric patients than in adult patients, but no age-specific associations with hemorrhage or survival outcomes were observed (Figure 4, panel A).

The acute-phase response refers to a constellation of host responses that occur during infection and other inflammatory processes (*14*). These responses are classically triggered by proinflammatory cytokines and lead to increased levels of acute-phase reactants. These markers of inflammation are often used clinically to assist in diagnosis and to track a patient's response to therapy in many infectious or inflammatory processes. Of the acute-phase reactants that we measured—SAA, C-reactive protein, ferritin, and IgG—only SAA and IgG levels demonstrated age-specific associations. SAA levels were higher for pediatric patients than for adult patients and were higher at later times of infection (6–15 days after symptom onset) for pediatric patients without hemorrhagic manifestations than for those with hemorrhagic manifestations (Figure 2, panel A). Total IgG levels were higher for samples from pediatric patients than for those from adult patients (Figure 4, panel B), but no age-specific associations with death or hemorrhage were observed for this biomarker.

Biomarkers of Endothelial Function

Given the role of the endothelium in maintaining vascular integrity and modulation of hemodynamic stability and the vascular leakage seen in EVD, we included several markers of endothelial function in our study (sICAM, sVCAM, and soluble E-selectin). sICAM and sVCAM demonstrated an age-specific association. ICAM and VCAM are expressed on endothelial cells and upregulated in response to proinflammatory cytokines. Both factors are shed from the surface of activated endothelial cells and can be measured in their soluble form in the serum (*15*). At 0–10 day after symptom onset, pediatric patients had higher levels of sICAM and sVCAM than did adults, and pediatric patients who died had higher levels of both factors than did those who survived (Figure 1, panels D, E).

Biomarkers of Coagulopathy

The frequent presence of hemorrhagic manifestations in EVD patients and the increased frequency seen in our pediatric population warranted an examination of the measureable factors that control coagulation and fibrinolysis. We measured PAI-1, fibrinogen, tissue plasminogen activator, D-dimer, thrombomodulin, and TF in all patient samples. PAI-1 levels were elevated in pediatric patients, and more so in those who died and those who had hemorrhagic manifestations (Figure 1, panel F; Figure 2, panel B). TF levels were slightly elevated in pediatric patients (Figure 4, panel C), but no age-specific associations with hemorrhage or death were shown.

Discussion

Differences in disease severity for patients of different ages are not uncommon in infectious diseases. For example, tuberculosis is associated with disseminated disease in children <5 years of age and focal pulmonary disease in adults but causes infrequent and mild disease in school-aged children and adolescents (*16*). A similar pattern was observed in this evaluation of patients infected with SUDV.

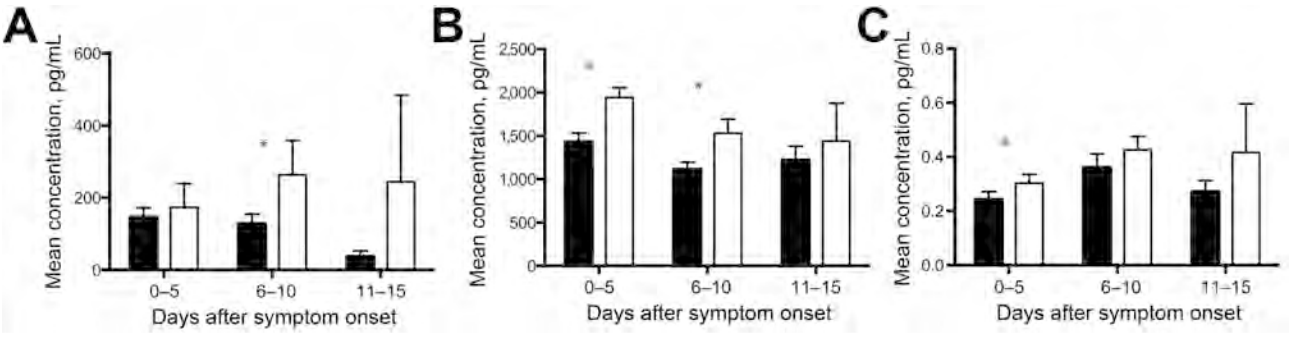

Figure 4. Biomarkers associated with age but not survival outcome or hemorrhage for patients with Ebola virus disease: black, adult; white, pediatric. A) Macrophage colony-stimulating factor; B) total IgG; C) tissue factor. Mean levels are depicted in each patient group as a function of time after symptom onset. Error bars represent SE; * indicates $p \leq 0.05$. Numbers of specimens included in each group are as follows: adult at 0–5 days, 47; adult at 6–10 days, 46; adult at 11–15 days, 19; pediatric at 0–5 days, 25; pediatric at 6–10 days, 18; pediatric at 11–15 days, 6.

Two possible explanations for the increased rate of death among children <5 years of age are the contributions of co-occurring conditions or the immature immune systems in children of this age. We extensively assessed biomarkers in serum samples from this SUDV outbreak and found that IL-10, IP-10, RANTES, sICAM, sVCAM, and PAI-1 were higher in pediatric patients than in adult patients and were also associated with specific outcomes in pediatric patients.

RANTES was the only factor we studied that demonstrated an association with higher survival rates in children. A known chemoattractant for monocytes and T cells, RANTES is produced by many cell types, including endothelial cells and macrophages (*17*), and plays a role in activation and proliferation of antigen-specific T cells (*18*). Decreased levels of RANTES in adult patients infected with chikungunya virus and in children infected with respiratory syncytial virus have been associated with more severe disease (*19*,*20*), and lower RANTES levels in children have been associated with death from cerebral malaria (*21*). RANTES$^{-/-}$ mice infected with lymphocytic choriomeningitis virus show decreased CD8+ T cell cytokine production and cytotoxic ability coincident with higher viral loads (*22*). Lymphocyte apoptosis has been seen in vitro in response to EBOV infection (*23*), and SUDV patients who died have had lower numbers of T cells, CD8+ T cells, and activated CD8+ T cells (*24*). CD8+ T cells were critical for EBOV survival in mouse models (*25*,*26*) and a nonhuman primate model (*27*). Therefore, it is not surprising that higher levels of RANTES were associated with survival in our study. The data from all of these studies suggest that, during SUDV infection, RANTES could recruit and activate T cells, leading to a stronger SUDV-specific T cell–mediated response and thus to improved survival. The occurrence of this phenomenon only in children is notable, but in models of familial hypercholesterolemia, the monoctytes of children, but not of adults, have increased RANTES expression (*28*), which suggests that children might have a greater capacity for RANTES production than do adults.

IL-10 levels were significantly elevated at the earliest times of infection in pediatric patients who died. The role of IL-10 in inhibiting antigen-stimulated T cell proliferation (*29*) supports the assumption that a T cell–mediated response is critical for survival during EVD. Levels of sICAM and sVCAM for children are normally higher than for adults (*30*), and the levels that we detected in surviving pediatric patients were consistent with these normal levels. Pediatric patients who died had sICAM and sVCAM levels 2–3 times above the reference range 0–10 days after symptom onset, but these levels dropped to within the reference range at 11–15 days. This pattern may reflect early excessive, and ultimately detrimental, endothelial activation in these patients. Consistent with this theory are the increased PAI-1 levels also seen in pediatric patients who died; PAI-1 is released by endothelial cells in response to activating cytokines (*31*).

IgG levels were higher in samples from pediatric patients than in samples from adult patients; this difference is notable because children usually have slightly lower levels of total IgG than do adults (*32*). The higher IgG levels might suggest a higher degree of immune activation, perhaps secondary to other infectious co-existing conditions, which are likely to be present in children living in a rural area of Africa. Consistent with this theory, high levels of malarial parasitemia have been associated with higher total levels of IgG in children in The Gambia (*33*).

We also observed associations between age and hemorrhagic manifestations for PAI-1 and SAA levels. Elevated PAI-1 levels in pediatric patients with hemorrhagic manifestations likely represent the overactive endothelium and not a functional inhibition of fibrinolysis, since PAI-1 activity is likely to be low, as it rapidly converts to the inactive form under physiologic conditions (*34*). Normal levels of SAA in children and adults are 10–90 ng/mL, and much higher levels can be seen in disease states (*35*). In

our study, higher SAA levels were seen in pediatric patients without hemorrhagic manifestations than in those with hemorrhagic manifestations. These levels were also higher than those seen in adults, regardless of the presence or absence of hemorrhagic manifestations. SAA is known to induce TF production by monocytes (*36*); we found that levels of TF were higher in pediatric patients , but these levels were not outside the normal range for adults or children and were not associated with hemorrhage or its absence. SAA treatment of endothelial cells causes decreased production of nitric oxide (NO) synthase and decreased NO bioavailability (*37*); elevated SAA levels in pediatric patients could have been associated with decreased levels of NO, and, therefore, with decreased vasodilatation and hemorrhage. However, the juvenile endothelium has decreased responsiveness to NO (*38*) and increased levels of circulating asymmetric dimethylarginine, an inhibitor of NO synthase (*39*); thus, altered levels of NO might not have functional consequences in pediatric patients. Alternatively, another, as yet undefined, function of SAA could be responsible for its association with pediatric patients who did not experience hemorrhagic manifestations. Finally, despite the association of PAI-1 with both hemorrhage and death, no statistically significant differences in survival rate were observed between patients with without hemorrhagic manifestations ($p = 0.23$). This finding suggests that these physiologic observations about hemorrhage are not causally related to survival.

An overactive endothelial response, as evidenced by elevated sICAM, sVCAM, and PAI-1 levels, was associated with death in children and adolescents. However, the adults in our study did not seem to be affected by this phenomenon. Co-existing conditions in the pediatric patients, such malaria or other childhood illnesses, could have contributed to endothelium reactivity, or this finding could be secondary to the known physiologic differences that exist between the adult and juvenile endothelium. Underscoring the importance of these age-specific endothelial differences, clinical trials using drugs that target these differences in treatment of cerebral malaria in children are underway (*40*).

In summary, our data suggest that different pathophysiologic mechanisms of disease may be at work in pediatric patients, and children may benefit from different treatment than their adult counterparts. Therapeutic interventions targeted at decreasing endothelial activation in pediatric patients early during the course of infection might include drugs that affect endothelial activation, such as statins. The clear association between survival and increased RANTES in pediatric patients also suggests that a better understanding of the mechanisms and molecular consequences of increased levels of this chemokine could be useful in future therapeutic design, especially in design of drugs that induce a stronger, earlier, antigen-specific T cell response.

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Increased Pyrethroid Resistance in Malaria Vectors and Decreased Bed Net Effectiveness, Burkina Faso

Kobié H. Toé, Christopher M. Jones, Sagnon N'Fale, Hanafy M. Ismail, Roch K. Dabiré, and Hilary Ranson

Malaria control is dependent on insecticides. Increases in prevalence of insecticide resistance in malaria vectors across Africa are well-documented. However, few attempts have been made to quantify the strength of this resistance and link it to the effectiveness of control tools. Using quantitative bioassays, we show that in Burkina Faso pyrethroid resistance in *Anopheles gambiae* mosquitoes has increased in intensity in recent years and now exceeds 1,000-fold. In laboratory assays, this level of resistance renders insecticides used to impregnate bed nets ineffective. Thus, the level of personal and community protection afforded by long-lasting insecticide-treated net campaigns will probably be reduced. Standardized methods are needed to quantify resistance levels in malaria vectors and link these levels to failure of vector control methods.

Long-lasting insecticide–treated bed nets (LLINs) have been shown repeatedly to provide protection against malaria transmission in Africa and reduce childhood mortality rates by ≈20% (*1*). Distribution of LLINs has increased over the past decade, and an estimated 54% of households at risk for malaria in sub-Saharan Africa have ≥1 LLIN. This factor has been a major contributor in reducing malaria incidence; the estimated malaria mortality rate for Africa has decreased by ≈49% since 2000 (*2*). These advances are now threatened by rapid selection and spread of resistance to insecticides in malaria vectors (*3*). Resistance to pyrethroids, the only class of insecticides available for use on LLINs, is now widespread in *Anopheles gambiae* and *An. funestus* mosquitoes, the major malaria vectors (*4*).

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To standardize monitoring for insecticide resistance, the World Health Organization (WHO) has developed simple bioassays that use filter papers impregnated with insecticide at a predefined diagnostic dose. A population is described as resistant to an insecticide if a mortality rate >90% is observed in these tests (*5*). These assays are useful for detecting resistance when it first appears in the population. However, these assays do not provide any information on the strength of this resistance. This information is crucial for assessing the likely effect of this resistance on effectiveness of vector control tools. The Global Plan for Insecticide Resistance Management in Malaria Vectors (*3*) recommends that all malaria-endemic countries monitor insecticide resistance in local vectors. However, because the correlation between results of diagnostic dose assays and control effectiveness remains undefined, simple detection of resistance in a mosquito population is not sufficient evidence to implement a change in insecticide policy.

In this study, we used variants of WHO assays and bottle assays of the Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) to quantify the level of pyrethroid resistance in a population of *An. gambiae* mosquitoes from Burkina Faso over a 3-year period. A high level of resistance was observed. The lack of comparator data from across Africa makes it impossible to conclude whether the pyrethroid resistance levels seen in Burkina Faso are atypical. However, these data should raise concerns for malaria control across Africa because we demonstrate that this level of resistance is causing operational failure of the insecticides used in LLINs.

Materials and Methods

The study site was in Vallée de Kou (Bama) in southwestern Burkina Faso, ≈25 km from the city of Bobo-Dioulasso. It consists of 7 small villages (area 1,200 hectares) and has been a major rice cultivation site since the 1970s. The area is surrounded by cotton-, rice-, and vegetable-growing areas in which insecticide use is intensive (*6*).

Multiple rounds of collections of third and fourth instar *Anopheles* spp. larvae were performed in a 1-km^2 radius from village 7 during June–July 2011, October 2011, June 2012, and July–October 2013. Mosquitoes from each collection round were pooled and reared to adults in insectaries at the Institut de Recherche en Sciences de la Sante/Centre Muraz in Bobo-Dioulasso or the Centre National de Recherche et de Formation sur le Paludisme (CNRFP) in Ouagadougou. Species were identified for a subset of mosquitoes from each collection round by using the Sine 200 PCR (*7*).

Non–blood fed *An. gambiae* female mosquitoes (3–5 days old) were tested with 5 insecticides in 4 insecticide classes: 0.75% permethrin (type I pyrethroid) and 0.05% deltamethrin (type II pyrethroid); 4% DDT (organochlorine); 0.1% bendiocarb (carbamate); and 1% fenitrothion (organophosphate) by using WHO susceptibility tests (*8*). Each batch of insecticide-impregnated papers was tested against mosquitoes of the *An. gambiae* Kisumu laboratory strain (insecticide-susceptible) at the CNRFP bioassay laboratory for quality control. Approximately 100 mosquitoes (4 replicates of 25 mosquitoes) were used per test (*5*). The average mortality rate and binomial confidence interval were calculated per insecticide (*9*).

In 2011 and 2012, the 50% lethality time (LT_{50}) for the VK7 strain of *An. gambiae* mosquitoes was determined by varying the length of exposure time (60–600 min). The mean mortality rate was recorded per time point, and the LT_{50} was estimated by fitting a logistic regression model by using logit-transformed probabilities (*10*) in R statistical software (http://www.r-project.org).

In 2013, CDC bottle bioassays were used to quantify the level of resistance to deltamethrin. Glass 250-mL bottles were coated with different concentration of deltamethrin ranging from 3.125 μg/mL to 125 μg/mL at CNRFP. Bottles were prepared according to CDC guidelines (*11*). Female mosquitoes (3–5 days) were aspirated into bottles for 1 h and subsequently transferred to insecticide-free paper cups for 24 h of observation. Four to six replicates were performed for each concentration and for the control bottles (impregnated with acetone). Equivalent age mosquitoes of the Kisumu strain were exposed to various insecticide concentrations (range 0.001 μg/mL–0.5 μg/mL). The 50% lethal dose (LD_{50}) was determined by using R statistical software.

A subset of LLINs that were distributed during the 2010 national distribution campaign were collected directly from houses in 2012; householders were given a new LLIN as a replacement. Only nets reportedly washed ≤5 times were included in the study. New net samples of the same type were also obtained from the population or from local markets. Six types of nets were tested: PermaNet 2.0 (deltamethrin coated on polyester; Vestergaard, Lausanne, Switzerland); Interceptor (α-cypermethrin coated on polyester; BASF, Florham Park, NJ, USA); DawaPlus (deltamethrin coated on polyester; TANA Netting Ltd., Bangkok, Thailand); NetProtect (deltamethrin incorporated into polyethylene; BESTNET, Kolding, Denmark); PermaNet 3.0 (deltamethrin coated on polyester with strengthened border side panels and deltamethrin and piperonyl butoxide incorporated into a polyethylene roof; Vestergaard); and Olyset (permethrin incorporated into polyethylene; Sumitomo Chemical Co., Ltd., Osaka, Japan).

Cone bioassays were performed according to WHO procedures (*12*) by using non–blood fed VK7 mosquitoes (3–5 days old) (obtained from larvae collection during October–December 2012) and Kisumu strain mosquitoes. Approximately 60 mosquitoes were assessed per net by using net samples from 2 sides and the top (20 mosquitoes/net sample). Mosquitoes were exposed to the insecticide for 3 min. Knockdown was recorded after 60 min, and the mortality rate was determined 24 h later. Mortality rates after exposure to each net were compared for wild-type and laboratory susceptible (laboratory raised) mosquitoes by using the Fisher exact test.

High-performance liquid chromatography was used to measure the insecticide content of 12 nets. Triplicate samples were tested from each net, and insecticide was extracted from five 8-cm^2 disks for each sample by vortexing them in acetone. A 10-mL aliquot was injected onto a reverse-phase, 250 mm, C18 column (Acclaim 120; Dionex, Sunnyvale, CA, USA). Separation was achieved by using a mobile phase of methanol/water (90:10 vol/vol) and at flow rate of 1 mL/min. Pyrethroid elution was monitored by absorption at 232 nm and quantified by peak integration (Chromeleon; Dionex). The quantity of pyrethroid insecticide was determined from a standard curve established with known concentration of pyrethroid insecticide.

Results

All *An. gambiae* VK7 mosquitoes collected were the M form, except for those collected during October 2011 and June–July 2013, of which the M form comprised 92% (315/335) and 90% (258/287) of the *An. gambiae* sensu lato populations, respectively. Susceptibility to 5 insecticides was assessed in adults emerging from VK7 strain larval collections in 3 successive years. *An. gambiae* mosquitoes remained fully susceptible to fenitrothion and showed a high mortality rate to bendiocarb (86.5% in June 2013) but low mortality rates to DDT (range 0%–3%) and for the pyrethroids deltamethrin and permethrin (range 1%–6%) However, no significant differences were found between results of the 3 successive years ($p = 0.055$) (Figure 1).

Initially, the strength of resistance was assessed by determining the LT_{50} for deltamethrin. In July 2011, an LT_{50}

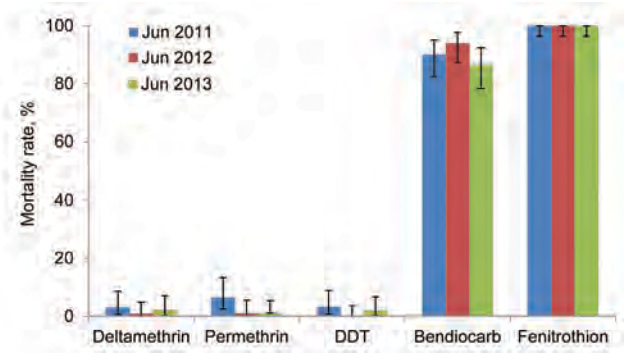

Figure 1. Results of World Health Organization (WHO) susceptibility tests for *Anopheles gambiae* VK7 mosquitoes, Burkina Faso. Adult female mosquitos were exposed to the WHO diagnostic dose of insecticides for 1 h, and mortality rates were recorded 24 h later. Error bars indicate 95% binomial CIs for 3 consecutive years (2011–2013) of sampling.

of 1 h 38 min (95% CI 1 h 34 min–1 h 42 min) was obtained but this value increased to 4 h 14 min (95% CI 3 h 53 min–4 h 36 min) in October of the same year (Figure 2), which is a 2.6-fold increase in only 4 months. An accurate LT_{50} could not be determined for samples collected in June 2012. The longest exposure time of 600 min (10 h) showed a mortality rate of 26% (95% CI 17.85%–35.50%), which extrapolates to an LT_{50} of 21 h 55 min (95% CI 14 h 3 min–34 h 14 min). The estimated LT_{50} for the Kisumu strain was <2 min (*13*). This time equates to resistance ratios in the field population versus the susceptible (laboratory raised) strain of 54- fold, 141-fold, and 730-fold in the 3 successive sampling periods.

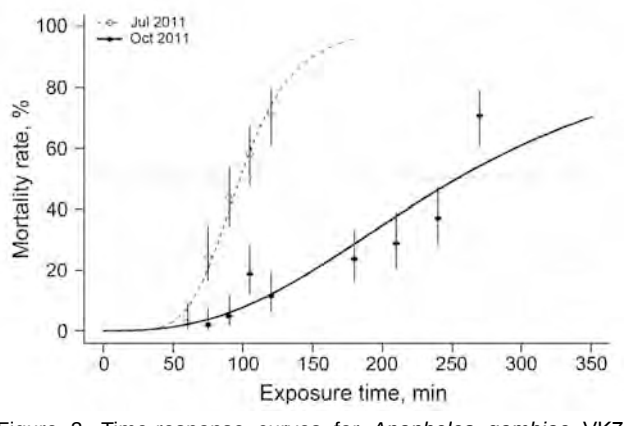

Figure 2. Time-response curves for *Anopheles gambiae* VK7 mosquitoes, Burkina Faso, July–October 2011. Adult females were exposed to 0.05% deltamethrin according to World Health Organization standard protocols. Time-response curves were fitted to data by using a regression logistic model and R software (http://www.r-project.org/). Dotted line indicates 50% mortality rate. Error bars indicate 95% binomial CIs for each time point. The 50% lethality times were 1 h 38 min for July and 4 h 14 min for October, which indicates an October:July resistance ratio increase of 2.6-fold.

Because the resistance level exceeded the threshold at which accurate LT_{50} levels were obtainable, in 2013, a variation of the CDC bottle bioassays was used to calculate the strength of resistance. Mosquitoes collected in July 2013 had an LD_{50} of 38.79 μg/mL (95% CI 32.99 μg/mL–46.06 μg/mL). The LD_{50} estimate for October was lower (21.55 μg/mL, 95% CI 15.77 μg/mL–31.22 μg/mL) and showed greater variation (Figure 3). By comparison, the LD_{50} for the insecticide-susceptible Kisumu strain calculated by using the same method was 0.021 μg/mL (95% CI 0.015 μg/mL–0.029 μg/mL). This value is equivalent to VK7:Kisumu resistance ratios of 1,847:1 for July and 1,026:1 for October.

The efficacies of 6 types of LLINs distributed as part of the National Malaria Control Program of Burkina Faso were assessed against the Kisumu and VK7 mosquito strains to assess the effect of resistance on LLIN effectiveness in a standardized WHO bioassay. New nets and nets that had been in use in the field for ≈2 years were assessed. Only 4 of 6 new nets showed 100% mortality rates against the Kisumu strain (Figure 4, panel A); a fifth net (Olyset) satisfied only the knockdown criteria. When used nets were tested, only 3 nets (Permanet 2.0, Permanet 3.0, and NetProtect) satisfied the WHO criteria (mortality rate ≥80% and knockdown rate ≥95%). When we evaluated the nets against VK7 mosquitoes, none of the nets satisfied the knockdown criteria and mean mortality rates were <50% for all new nets and used LLINs tested; mortality rates were lower for used nets than for all types of new nets (Figure 4, panel B).

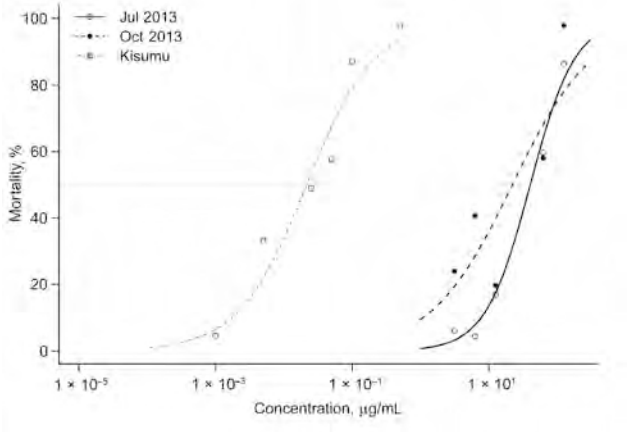

Figure 3. Dose-response curves for 3- to 5-day-old *Anopheles gambiae* VK7 female mosquitoes and Kisumu laboratory strain mosquitoes (insecticide-susceptible), Burkina Faso. Mosquitoes were exposed to different concentrations of deltamethrin in 250-mL glass bottles for 1 h. Dose-response curves were fitted to data by using a regression logistic model and R software (http://www.r-project.org/). Dotted line indicates 50% mortality rate. 50% lethality concentrations were 38.787 μg/mL (95% CI 32.993 μg/mL–46.062 μg/mL) in July 2013, 21.547 μg/mL (95% CI 15.771 μg/mL–31.223 μg/mL) in October 2013, and 0.021 μg/mL (95% CI 0.015 μg/mL–0.029 μg/mL) for the Kisumu strain.

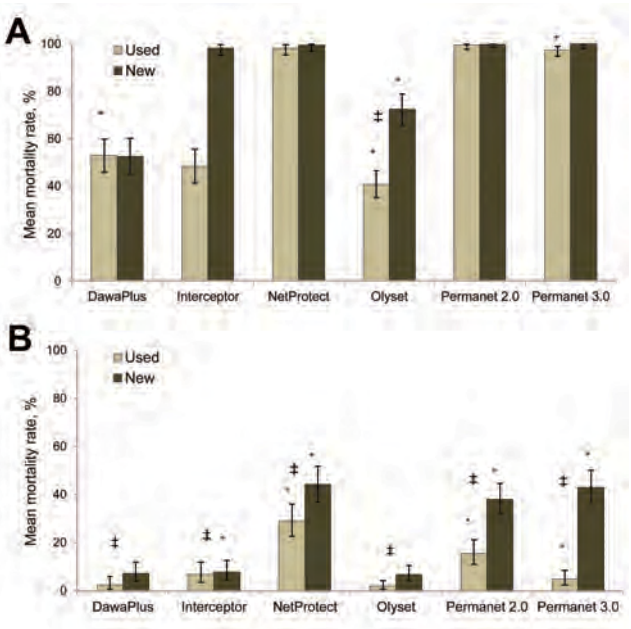

Figure 4. Mean mortality rates 24 h after exposure to new and used long-lasting insecticide–treated bed nets for A) *Anopheles gambiae* Kisumu laboratory strain mosquitoes (insecticide-susceptible) and B) *An. gambiae* VK7 mosquitoes, Burkino Faso. Error bars indicate 95% binomial CIs for the average of net type. *Indicates significant variation between independent nets of each type (p<0.05). ‡Indicates a significant difference between new and used long-lasting insecticide-treated bed nets (p<0.05).

Because PermaNet 3.0 nets have the synergist piperonyl butoxide incorporated into the net roof, we compared bioassay data for the roof and sides. Mortality rates were significantly higher when mosquitoes were exposed to the roof of the net when new nets were tested (p = 0.011) but not when used nets were tested (p = 0.20).

Low mortality rates observed in cone bioassays, even against susceptible mosquitoes, led us to investigate the amount of insecticide that could be extracted from LLINs used in the bioassays. Analysis by high-performance liquid chromatography was performed for net types that did not satisfy WHO criteria for susceptible mosquitoes; Permanet 2.0 was used as a control. We included data only for nets in which insecticide was coated onto the surface (rather than incorporated into the fibers) because the acetone extraction method used is not efficient in extracting insecticide from the fibers. In each case, higher levels of insecticide were extracted from new nets than from used nets (p<0.05, by 2-tailed *t* test) except for DawaPlus, for which the amount of active ingredient was ≈0 for used and new nets (Table 1). Less than 12% of the target concentration of insecticide could be extracted from LLINs that induced a mortality rate <80% in the Kisumu strain. In general, the expected concentration of insecticide was isolated from LLINs, which showed the target mortality rate of 80% for cone bioassays.

Discussion

Monitoring *Anopheles* spp. vectors for susceptibility to insecticides is recommended by WHO in all countries that use LLINs or indoor residual spraying for malaria control (*3*). Most countries in Africa that have implemented resistance monitoring since 2010 have detected pyrethroid resistance in some regions. Many countries have detected resistance to multiple insecticide classes (www.irmapper.com). Ideally, the first detection of resistance should elicit a change in insecticide class as part of a proactive resistance management program. However, because only pyrethroids are currently available for impregnating bed nets, and use of alternative insecticide classes for indoor residual spraying often result in higher program costs (*14*,*15*), resistance management options are severely limited. Thus, data obtained from routine resistance monitoring should be sufficient to make evidence-based decisions on insecticide-based vector control strategies. Use of only diagnostic dose assays alone can mask major changes in the strength of resistance. This finding can be seen in the current study, in which no major difference was seen in pyrethroid mortality rates over a 3-year period with a fixed exposure of insecticide. However, when exposure time or concentration was varied, increases in the strength of the resistance were observed.

We used 3 bioassays to quantify the strength of the resistance and link this strength to the effectiveness of current vector control tools. Each of these methods has limitations, which are summarized in Table 2. In this study, we did not use a tunnel test, in which mosquitoes are exposed overnight to a holed net, with a guinea pig as bait, and the

Table 1. Characteristics of insecticide extracted from long-lasting insecticide–treated bed nets, Burkina Faso*

Net type	Condition	No. tested	Active ingredient concentration, mg/m^2 (SD)	Target concentration, mg/m^2	Mortality rate, %, for *Anopheles gambiae* Kisumu laboratory strain mosquitoes (95% CI)†
DawaPlus	Used	3	0	80	52.43 (45.37–59.41)
DawaPlus	New	2	1.31 (1.60)	80	51.30 (41.80–60.73)
Interceptor	Used	3	22.2 (18.56)	200	48.48 (41.30–55.67)
Interceptor	New	2	223.95 (43.58)	200	97.65 (93.30–99.51)
PermaNet 2.0	Used	2	66.3 (1.82)	55	100.00 (97.02–100.00)

*Active ingredient concentration was determined by high-performance liquid chromatography. Mortality rate was determined after a 3-min exposure.
†Insecticide susceptible.

Table 2. Alternative field bioassay methods for assessing strength of insecticide resistance*

Method	Brief description	Advantages	Disadvantages
Time-response curves (LT_{50})	Exposure to fixed concentration of insecticide for varying periods	Can be performed by using diagnostic dose filter papers available from WHO; simple to perform	Not appropriate for highly resistant mosquito populations in which long exposure times required
Dose-response curves (LD_{50})	Exposure to varying concentrations of insecticide for a fixed period	Can be readily adapted for populations of different resistance status by varying concentration of insecticide used	Challenging to accurately measure small quantities of insecticide needed for some pyrethroid insecticides; if bottles are reused, stringent washing conditions are needed
Cone bioassays on treated surfaces	Exposure to field dose of insecticide for fixed period	Concentration of insecticide being evaluated is the field dose	Mosquitoes can avoid exposure by resting on side of cones, particularly for new preparations of some pyrethroids.

*For all assays, use of age-standardized mosquitoes and inclusion of a 24-h recovery period to capture metabolic resistance mechanisms (which can be slower to act) is recommended. LT_{50}, 50% lethality time; WHO, World Health Organization; LD_{50}, 50% lethal dose.

mortality rate and blood-feeding inhibition are measured (*12*). It would be useful to determine if the longer exposure time used in the tunnel test resulted in higher mortality rates, although given the long exposure time required to achieve the LT_{50} with impregnated papers, it is expected that the LLINs would also fail a tunnel test against the VK7 mosquito population.

There is a need for agreement on a consensus method for resistance monitoring, together with clear guidelines for interpreting the operational value of the results and recommended courses of action. This method would not necessarily replace diagnostic dose assays, which are valuable for detecting the prevalence of resistance in a population, but would instead provide a quantitative estimate of the strength of resistance that is linked to predicted control failure. Such assays are common practice in the agricultural sector and a cross-sectorial approach would be invaluable for improving resistance monitoring in malaria control.

The resistance levels we report in the current study are alarming. Because few studies have attempted to quantify resistance strength in field populations, it is difficult to know if this extreme resistance phenotype is exceptional or symptomatic of the status of pyrethroid resistance in malaria vectors in Africa. Two other studies have used the LT_{50} method to assess the strength of resistance to pyrethroids in field populations compared with susceptible (laboratory raised) strains. In 2011, deltamethrin resistance ratios of 138-fold were recorded in Tiassalé, Côte d'Ivoire (*13*) and 292-fold in Jinja, Uganda (*16*). Thus, to our knowledge, deltamethrin resistance levels of 730-fold in 2012 (estimated by LT_{50}) and >1,000 fold in 2013 (estimated by LD_{50}) reported in the current study are the highest in the published literature.

This level of resistance will almost certainly affect the effectiveness of vector control. We demonstrate that the insecticide resistance of VK7 mosquitoes severely affected the performance of LLINs in standardized laboratory bioassays. In Kenya, pyrethroid-resistant mosquitoes were found resting inside holed LLINs and, when tested by cone bioassays, these LLINs were also found to be ineffective at killing local vectors (*17*). Linking resistance strength with increases in malaria transmission is currently not possible but is a key priority for further studies. No data on the strength of pyrethroid resistance in *An. funestus* mosquitoes in southern Africa in 2000 are available. This resistance has been widely accredited with causing control failure that resulted in a dramatic increase in malaria cases (*18*).

Finally, it is vital to recognize that insecticide resistance is not the only cause of reduced effectiveness of vector control tools. In the current study, we showed that cone bioassays for new and used LLINS were less effective at killing the field-caught *An. gambiae* mosquitoes than they were against a standard susceptible (laboratory raised) strain, which provided additional evidence for the effect of resistance. However, we also found that 2 brands of the LLINs (Olyset and DawaPlus) showed poor performance against the susceptible mosquito strain, and another LLIN (Interceptor) showed adequate performance only when new nets were used. Although these data were obtained for a small sample set, they are a cause for concern and must be investigated further.

K.H.T., C.M.J., and H.M.I performed the experiments and analyzed the data; C.M.J. and H.R. designed the study; K.H.T., C.M.J., and H.R wrote the manuscript; and S.N. and R.K.D. supervised the field work.

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Mr Toé is a doctoral student at the Liverpool School of Tropical Medicine, Liverpool, UK. His research interests are evaluating the distribution, causes, and effects of insecticide resistance in malaria vectors in Africa.

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Malaria Control and Elimination,[1] Venezuela, 1800s–1970s

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Venezuela had the highest number of human malaria cases in Latin American before 1936. During 1891–1920, malaria was endemic to >600,000 km^2 of this country; malaria death rates led to major population decreases during 1891–1920. No pathogen, including the influenza virus that caused the 1918 pandemic, caused more deaths than malaria during 1905–1945. Early reports of malaria eradication in Venezuela helped spark the world's interest in global eradication. We describe early approaches to malaria epidemiology in Venezuela and how this country developed an efficient control program and an approach to eradication. Arnoldo Gabaldón was a key policy maker during this development process. He directed malaria control in Venezuela from the late 1930s to the end of the 1970s and contributed to malaria program planning of the World Health Organization. We discuss how his efforts helped reduce the incidence of malaria in Venezuela and how his approach diverged from World Health Organization guidelines.

Venezuela had the most human malaria cases in Latin America before 1936. During 1891–1920, malaria was endemic to >600,000 km^2 of Venezuela; deaths from malaria substantially reduced the population during 1891–1920 (*1*). No pathogen, including influenza virus (1918 pandemic), caused more deaths than malaria during 1905–1945. Early malaria epidemics had mortality rates of 60–70 deaths/1,000 persons; rates were as high as 531 and 1,125 deaths/100,000 persons in Carabobo and Cojedes States in 1941 (*2*).

Venezuela can be divided into 3 zones: central (Los Llanos; plains), southern (Guayana), and northern (Costa-Cordillera; coast–mountain range) (Figure 1). Los Llanos has grassy plains intersected by rivers that flood and abut jungles. This zone contains 36% of Venezuela and bodies of still water in which vectors breed. In the early 20th century, 20% of the population lived in Los Llanos and had the greatest malaria prevalence; however, no large epidemics occurred there. In northern regions, malaria was considered hyperendemic based on spleen indexes (Table), which occasionally reached 100. In southern regions, spleen indexes were <50. The malaria vector was *Anopheles darlingi* mosquitoes, one of the most efficient neotropical vectors. In Venezuela, these mosquitoes bite throughout the night or adapt to human behavior. Their larvae require clear water (*5*). These mosquitoes were absent in southwest regions near the Apure River, which were free of malaria (*3*).

Guayana, which borders Brazil, Colombia, and Guyana, has a tropical forest, patches of open country in northern regions, and a savannah plateau in southern regions. Although Guayana contains 46% of Venezuela, in the early 20th century, it contained only 3% of the population, which was concentrated around urban centers. Malaria cases typically occurred at altitudes of 500–1,000 m on the plateau, where *An. darlingi* mosquitoes predominated. Spleen indexes were usually <50. In northeastern regions, *An. darlingi* mosquitoes were absent and spleen indexes were ≈5. The southwestern border of Guayana and Colombia was free of malaria because the tannic Atabapo and Guainía Rivers kept riverine villages free of *An. darlingi* mosquitoes (*3*).

Costa-Cordillera, a coastal plain that abuts mountains, contains 18% of Venezuela. Before the 1940s, malaria epidemics followed a 5-year cycle associated with coastal invasions by *An. darlingi* mosquitoes. These cyclic increases in malaria continued until at least 1997 because of the El Niño Southern Oscillation (*6*). Early control efforts divided Costa-Cordillera into western, central, and eastern sectors. The eastern sector contained Nueva Esparta State, Caribbean islands, and Sucre State. The central sector contained valleys and mountains. The western sector contained valleys of Lake Maracaibo and the Andes Mountains (*3*).

In the 1940s, 70% of the population of Venezuela lived in Costa-Cordillera (*6*). During the 1940s and 1950s, the greatest malaria endemicity occurred where *An. darlingi* mosquitoes predominated, although rates were also high where *An. albitarsis* mosquitoes predominated. Regions

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[1]During much of the period reported in this paper, the historically accurate term was eradication.

with moderate endemicity typically had *An. albimanus* mosquitoes, which contributed to epidemics associated with heavy rainfall or rice cultivation because larvae require sunlit water (*2*,*5*). Coastal marshes precluded survival of *An. darlingi* mosquitoes but supported survival of *An. albimanus* mosquitoes that were tolerant of brackish water (*5*). At higher altitudes, but rarely above 500 m, malaria was transmitted by *An. pseudopunctipennis* mosquitoes (limit 1,000 m) (*3*).

In eastern Costa-Cordillera, *An. darlingi* and *An. albimanus* mosquitoes predominated in western Sucre, and *An. aquasalis* mosquitoes predominated in eastern Sucre (*3*). *An. aquasalis* mosquitoes are typically coastal vectors because they compete poorly with other *Anopheles* species and have limited predator defenses (*5*). These mosquitoes are exophilic, prefers to live outdoors, and are refractive to domicile insecticide spraying (*7*). In central Costa-Cordillera, *An. darlingi* mosquitoes were found near Lake Valencia, which had the greatest malaria prevalence. In the southern sector, *An. nuneztovari* and *An. pseudopunctipennis* mosquitoes were present in foothills and *An. albimanus* mosquitoes were present in a valley (*3*). *An. nuneztovari* mosquitoes were exophilic and resisted domicile DDT spraying (*8*). In the western sector, *An. albimanus* mosquitoes predominated in northern low-rainfall zones, and *An. darlingi* mosquitoes predominated in high-rainfall zones (*3*).

Before DDT use, most municipalities in central Costa-Cordillera and western Los Llanos had endemicity ratios <8 (Table) and mortality rates of 20–25 deaths/1,000 persons. Some municipalities had endemicity ratios of 10–15 and mortality rates of 30–50 deaths/1,000 persons (*2*).

History of Malaria in Venezuela

During the Venezuelan War of Independence (1820s–1830s), a malaria epidemic affected armies in Los Llanos (*9*). In August 1879, Ortiz in Los Llanos reported 125 malaria cases and ≥2 deaths among ≈9,600 inhabitants. Witch doctors and charlatans complicated disease treatment. In 1880, a total of 127 cases were reported, but the number of cases gradually decreased until at least 1885 (*10*). Epidemics occurred in Ortiz during 1890–1891 (*9*).

In 1894, Dr. Santos Aníbal Dominici identified the malaria parasite in patients at Vargas Hospital in Caracas (*11*,*12*). The National Health Office and Institute of Hygiene and Chemistry, Bacteriology, and Parasitology Laboratories opened in 1911; a National Health Act was promulgated in 1912 (*12*). During the 1920s, quinine was freely distributed in some regions (*9*).

In 1926, the National Health Office began to study malaria around Lake Valencia with support from the Rockefeller Foundation. The office conducted a malaria survey during 1927–1928 and recommended spraying Paris green, draining lagoons, and cultivating surrounding

Figure 1. Three zones of Venezuela used by Arnoldo Gabaldón for treatment of malaria: A) Costa-Cordillera, B) Los Llanos, and C) Guayana (*3*).

fields (*9*,*13*). Malaria was widespread in Los Llanos, the lower Yaracuy Valley, and the Lake Maracaibo District, but not in the Caracas Valley or the coastal region near La Guaira. Epidemics occurred in sections of the Lake Valencia basin (*14*).

The Rockefeller Foundation started a 1-year study of malaria around the Maracay District of Lake Valencia, which included patient histories, and spleen, blood, and vector surveys. Interventions began almost simultaneously, which decreased malaria cases and quinine use. A permanent program was recommended, which included better drainage for wells, irrigation ditches, and sewers (*14*). In 1930, malaria cases increased in Maracay because of introduction of *An. darlingi* mosquitoes. In 1 area, all 500 inhabitants were infected with *Plasmodium falciparum* (*15*). Cooperation with the Rockefeller Foundation lapsed in 1932, possibly because of a backlash against foreign oil companies (e.g., the Rockefellers' ownership of Standard Oil) (*9*,*12*,*16*). Despite the foundation's absence, effectiveness of antimalarial treatments was studied in Guárico in 1935 (*17*).

In 1936, Dr. Enrique Tejera, formerly manager of the National Health Directorate's Bacteriology and Parasitology Laboratory, became Minister for Health and Social Assistance. The ministry oversaw the Malaria Division, which had a budget of ≈$10 million (in 2014 US dollars). Tejera created a national public health system based on administrative medical, research, and control technique units, as advocated by the Rockefeller Foundation and the League of Nation's Malaria Commission. He established agreements with the foundation and a scholarship program for persons from Venezuela to study at universities in the United States (*12*,*18*,*19*).

Table. Commonly used malaria terms, Venezuela

Term	Definition
Spleen index or spleen rate	Point prevalence of persons with a palpably enlarged spleen (splenomegaly), which is strongly associated with malaria in many countries, including India and Venezuela. Although this term is not an exact measurement of malaria infection, it is considered an indicative public health measurement in tropical countries. The reference measurement in Venezuela was 5%, where malaria was not present (*1*).
Endemicity ratio	Lowest spleen index observed in a 5-year period divided by 5, which is the assumed reference value for this index. This ratio was complemented by the ratio of epidemicity, in which the numerator was replaced by the greatest spleen index over a 5-year period (*2,4*).
Index of infection	Prevalence of persons with malaria infection as determined by existing measurements, such as spleen indexes or blood smears.

In 1936, the Law on the Defense against Malaria was modeled on laws in Argentina and passed. The law acknowledged the national threat of malaria and described comprehensive interventions at local to national levels (*20*). According to dissatisfied physicians, including Tejera, the law proposed insufficient scientific studies to inform officials on whether malaria should be controlled or eradicated. Tejera resigned rather than ratify this law. Dominici took over and designated Gabaldón as Director of Malariology (*11,12*).

The Gabaldón Era

Gabaldón, a physician, had assisted Tejera at the National Health Directorate's laboratory during 1928–1930. He had then studied at the German Institute of Naval and Tropical Diseases and the Italian Experimental Station for the Antimalarial Battle before returning to Venezuela in 1932. He received a health science doctorate from John Hopkins University in 1935 through the Rockefeller Foundation and interned at Rockefeller University in New York City (*12*).

Under Gabaldón, the Malaria Division opened in 1936. The division had 4 sections: Epidemiology, Local Malaria Control and Quinine Distribution Commissions, Malaria Engineering, and Administration (*18*). The Malaria Division conducted an epidemiologic evaluation of malaria, vectors, and habitats and found that malaria was present throughout Venezuela (Figure 2, panel A) (*21,22*). It established a School of Malariology in 1937 in Maracay (*9,19*) and trained federal and state malaria staff, including doctors, inspectors, and engineers, during the 1940s (*9,19*). It also hosted the annual International Malaria and Environmental Health Course for New World malariologists (*9*).

The initial goal of the Malaria Division was to define where to apply malaria control by creating village-level maps and monitoring fumigation crews. Inspectors later managed personnel in rural areas who provided municipal diagnosis. Personnel were selected based on education and community status (*19*).

In 1937, field stations were established in towns and rural districts to monitor malaria incidence (*1*). Volunteers provided free quinine and quinacrine tablets every 7 days to febrile citizens (*1,2*). Blood films were examined and vectors identified at field laboratories and results were verified at central laboratories (*1*). In 1 year, 800,000 persons were treated (*2*). By 1941, the division had surveyed 8 states and planned to examine the remaining 12 states by December 1942 (*1*).

Vector control consisted of implementing sanitary engineering, including paving canals with concrete (some towns required >50 km of paving), and applying insecticides and larvicides (Paris green and pyrethrum), especially during epidemics (*2*). Larvicides were impractical without drainage to limit vector-breeding areas. Mosquito nets were widely distributed (*1*). Vector control was limited to urban areas because rural control was not economical (*1*). The main vectors were *An. albimanus* and *An. darlingi* mosquitoes, although *An. darlingi* mosquitoes were eliminated from some towns (*2*).

Gabaldón successfully experimented with pyrethrum spraying in 1940. By 1941, malaria control had been implemented in 10 cities and the index of infection (Table) was 0 in Maracay (*18*). During 1945, Gabaldón visited the United States and learned about DDT. He procured 10 kg with the support of Tejera in his capacity as governor of Carabobo. In December 1945, DDT domiciliary spraying began on a ranch in Morón, Carabobo, and eventually included 80 houses (*9*).

Indoor spraying with DDT was planned for the malarious region without preliminary trials, although initially only in northern and central Venezuela (*2*). DDT was secured through Colonel Ernest Steel, director of the Inter-American Cooperative Office of Public Health (*9*). Spraying was conducted simultaneously with antimalarial programs by using a volunteer network (*1*). Initially, 1 g of DDT/m^2 was applied every 3 months, then every 4 months; 2 g was then applied every 6 months (*2*). Random wall scrapings were taken to verify proper spraying (*1*).

Other insecticides were also used for spraying homes. These insecticides included a benzene hexachloride/DDT mixture in areas heavily infected with triatomids. Spraying with DDT continued through a trial and error phase until the entire malarious region was covered in 1951. Challenges included weather, uneducated workers, poor supervision and transportation, and developing a team spirit (*2*). In 1946, Rockefeller University was invited to undertake malaria studies with the Malaria Division and opened a research laboratory in Maracay that focused on residual insecticide effectiveness (*23–25*).

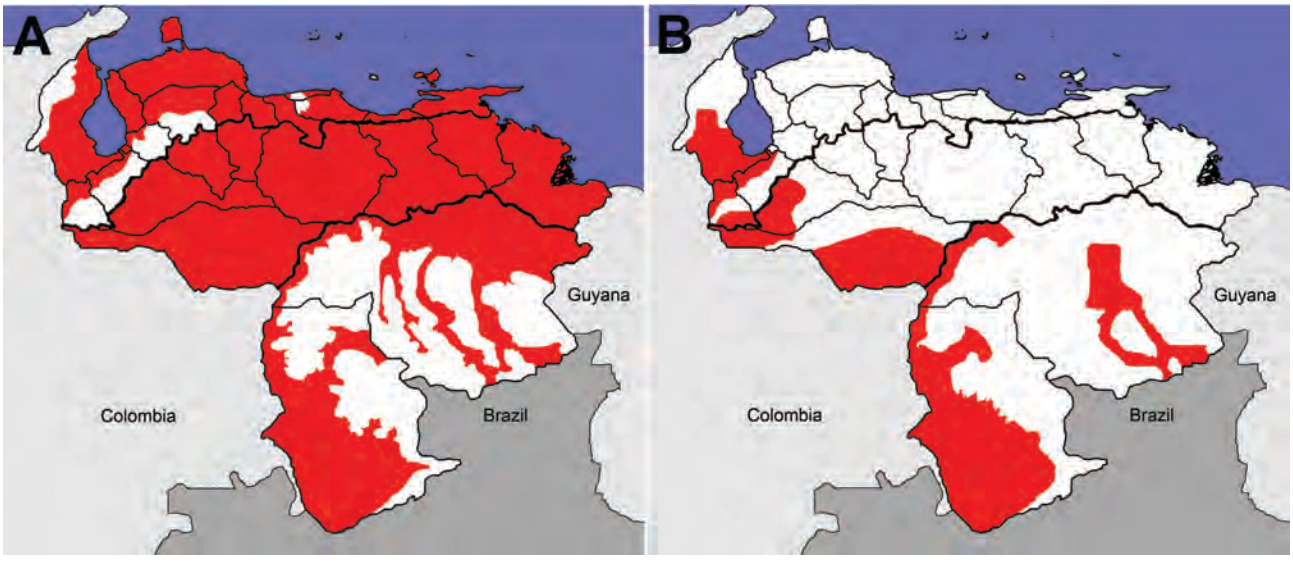

Figure 2. Distribution of malaria (red) in Venezuela during A) 1937 and B) 1980. This figure is qualitative because the authors did not have direct access to underlying data from original sources (*1*,*21*).

Success of DDT spraying was startling. Malaria disappeared after 3–5 years without additional measures beyond occasional quinacrine use in areas where *An. darlingi* and *An. albimanus* mosquitoes predominated. The populace was stationary, which limited introduced cases and facilitated eradication. (For the remainder of the paper, the term malaria eradication, rather than the modern term malaria elimination, will be used because eradication was public health terminology for the historical period described.) Eradication in eastern regions was slower because of *An. aquasalis* and *An. nuneztovari* mosquitoes (*1*). *P. falciparum* malaria was most common, although *P. vivax* malaria predominated among children <5 years of age (*2*).

In 1946, Gabaldón proposed an Expert Committee on Malaria to the Interim Commission that would suggest future work by the World Health Organization (WHO) (*1*). Successes in Venezuela and India led the committee to conclude that "insecticides can be [used]… for a widespread attack on malaria with… a significant reduction of morbidity" (*26*). The Expert Committee defined malaria control guidelines in 1947 (*27*). Gabaldón chaired the WHO expert committee meeting and attended nearly all of its first 15 sessions (*28*).

A malaria-eradication public health network was developed by the 1950s, which WHO used as an eradication program model (*2*,*19*). By 1952, there were 590 physicians throughout central Costa-Cordillera. Physicians reported clinically diagnosed malaria cases each week to the Division of Epidemiology and Vital Statistics. The division chief also sent a letter to physicians explaining the role of the malaria eradication network and likening cases to those of yellow fever or plague (*2*).

The division emphasized microscopy confirmation of blood film findings, and medical dispensaries paid for blood films in some regions. Thick and thin blood films were examined at field laboratories and at the central laboratory, where all positive results and 10% of negative results were verified. In rural districts, after domicile pesticide spraying, health care workers obtained blood films from febrile patients or persons who had been febrile in the past week. Films were used only when physicians were absent, although the index of infection for health workers (0.3%) was nearly the same as that for case-patients (0.2%) in 1952. When malaria occurred where it been declared eradicated, an inspector and survey team measured adult vector and larval densities in areas of 5–10 km around reported case-patients. Houses were resprayed if >3 months had passed since workers' last visit (*2*).

In the first 8 years of DDT spraying, *An. darlingi* mosquitoes and endemic and epidemic malaria were eradicated from central Costa-Cordillera, where ≈50% of the population of Venezuela lived. However, *An. aquasalis* mosquitoes were not eliminated from coastal Costa-Cordillera (0.1% of the malarious zone), where 28% of the malaria cases in Venezuela occurred (*1*). Another 50.6% of malaria cases occurred on the western border of Costa-Cordillera and Colombia (3.3% of the malarious zone), where vectors were *An. darlingi*, *An. nuneztovari*, and *An. pseudopunctipennis* mosquitoes (*1*). Control efforts were successful except near forests and banana plantations (*7*). Northern Costa-Cordillera bordering Colombia (5% of the malarious zone) had 5.6% of malaria cases, and vectors were *An. albimanus* and *An. darlingi* mosquitoes (*1*).

Eradication was not attempted in areas where distances inhibited economic control or outdoor transmission predominated. These areas included northern Costa-Cordillera along the border with Colombia, Apure and Delta Amacuro in Los Llanos, and Bolivar and Amazonas in Guyana (*2*). In Los Llanos and Guayana, 56.6% of the malarious zone contained 14.7% of malaria cases in 1952, and the main vectors were *An. albimanus*, *An. albitarsis*, and *An. darlingi* mosquitoes (*2*).

By 1954, malaria had been eliminated or was decreasing across 30% (≈180,000 km^2) of the malarious zone (*2*). Gabaldón wrote that Venezuelan "malaria eradication… will be attained in the near future… [with]… two exceptions…. the first… two small areas… [with] out-of-doors transmission… the second… districts inhabited by nomadic and… wild Indian tribes, most… in Amazonas, Apure, Bolívar, and the Delta Amacuro" (*2*). Gabaldón refuted critics by citing successes in Argentina, Ecuador, the United States, and Venezuela (*28*). The hope was to eradicate malaria by 1955 (*29*).

However, in 1956, Gabaldón insisted that "nothing except the lack of funds should prevent the attainment of [a malaria-free Venezuela]" (*29*). Malaria reached its lowest incidence in 1959 (911 cases), and 68% of the malarious zone (407,945 km^2) was malaria free (*1*,*21*). Gabaldón was Minister of Health during 1959–1964 and changed the Division of Malariology to the Ministry of Malariology and Environmental Health, which now included the divisions of sanitary engineering, rural water supply, rural housing, and ankylostomiasis and other helminthic diseases. He also suggested, through the Pan American Sanitary Bureau, that WHO create a registry of regions where malaria eradication was achieved (*28*).

The results of DDT spraying illustrated that primary vectors could mask contributions of secondary vectors (*28*). Spraying eliminated *An. darlingi* mosquitoes, but other vectors continued to transmit malaria (*2*). Before use of DDT, the most prevalent *Plasmodium* species was *P. falciparum*, followed by *P. vivax*, and *P. malariae*. Almost 40 years after introduction of DDT, *P. vivax* predominated; there was little *P. falciparum* and no *P. malariae* (*1*). In control areas, demand for quinacrine decreased. There were fewer malaria-positive blood films and death certificates that mentioned malaria or fever, and lower overall mortality rates, especially among young persons (*1*).

In 1959, febrile patients whose blood films were positive for *P. falciparum* were treated with chloroquine, followed by 4 weekly doses of chloroquine and pyrimethamine. *P. vivax* and *P. malariae* malaria was treated with chloroquine and primaquine for 3 days, then with primaquine for 11 days. Primaquine treatment was interrupted if side effects developed, and it was not given to persons >4 months of age. In the presence of DDT-refractory vectors, persons were treated with suppressive weekly or biweekly doses of pyrimethamine. Chloroquine was substituted in areas where *Plasmodium* spp. were pyrimethamine resistant (*30*).

In 1961, WHO declared malaria eradicated from 68% (407,945 km^2) of the malaria zone in Venezuela (*1*,*31*). However, the DDT campaign ended in 1965 without eradicating malaria (*2*,*22*). Gabaldón's successes enabled him to ignore WHO malaria strategies developed during meetings he chaired. In 1968, a WHO report found that "the concept of malaria eradication adopted by the national authorities has… and is… at variance with the [expert committee]." Against committee recommendations, Gabaldón had enlarged the eradication program to address other public health issues and no longer conducted active case detection in maintenance zones, except near zones in the attack phase. Health service staff did not view eradication as integral and were inadequately supervised. Active case detection was no longer conducted in most locations, and with passive case detection, only 30%–35% of blood films were examined, Since 1960, a total of 94 of 385 municipalities had not prepared blood films (*28*).

Venezuela declared that malaria was eradicated in some regions, although insecticide spraying continued. This declaration was in conflict with the WHO eradication definition because spraying could indicate residual endemicity. Gabaldón claimed that spraying prevented reintroduction. WHO resolved this disagreement by declaring that Venezuela was a special case of malaria eradication because it occurred before the 1960 WHO definition was developed (*28*).

Gabaldón proposed that WHO revise its global eradication strategy and include his strategies at the 1970 expert committee meeting. He concluded that permanent interruption of transmission was unachievable because of relapses and new introductions. However, if initial cases were discovered early, elimination measures could be applied without altering overall malaria eradication status (*28*). This proposal reflected his earlier shift from eliminating the reservoir of infective cases to interrupting transmission through domicile insecticide spraying and killing engorged mosquitoes (*1*). Insecticide spraying was a natural measure and applied seasonally even without adequate supervision (*28*).

Gabaldón suggested 2 levels of malaria reintroduction prevention: first-degree, which sought to prevent vector and parasite importation by proactively searching for carriers; and second-degree, which focused on limiting reestablishment of endemic malaria transmission, chiefly through pesticide spraying (*28*). He later said that first-degree prevention was ineffective and costly when applied to infected agricultural workers who moved from malarious regions to malaria-free regions (*1*). There was no need for first-degree prevention if second-degree

prevention was maintained. Therefore, resources required for first-degree prevention were better spent in Latin America on permanent public health programs for transmission control, combined with preventive medicine and environmental improvement. Instead of dismantling eradication infrastructure, Gabaldón suggested that it should be converted into "vector-borne-disease control… in charge of problems that require… control measures… among environmental health activities" (*28*).

After the expert committee rejected Gabaldón's revisions, he disassociated himself from parts of the WHO 15th report (*28*,*32*). His first-degree and second-degree prevention were mentioned. However, his assertion that second-degree prevention obviated the need to visit carrier households was not mentioned. The report emphasized integration of malaria control with health services. Gabaldón had integrated malaria eradication with preventive medicine and environmental sanitation and believed that adding medical services would be cost prohibitive. Finally, the report suggested that a region must abstain for 2 years from large-scale insecticide or mass treatment to go from the consolidation phase to the maintenance phase of eradication (*32*). Gabaldón later blamed this recommendation as the principal cause of renewed transmission in tropical countries where spraying had been correctly applied (*1*).

In 1971, the malaria-free region of Venezuela had increased to 77% (460,054 km^2) of the malarious zone. Malaria control in malarious regions consisted of domicile spraying with DDT every 4 months (no agricultural use), as had been implemented since 1947 and would continue until 1983 (no insecticide resistance). It also included weekly mass administration of chloroquine and primaquine for <3 months in villages with monthly parasite incidences >50 per 1,000 (units were not provided) (*1*). Primaquine was probably well received because only 2% of persons sampled in Caracas in 1966 had the glucose-6-phosphate dehydrogenase deficiency associated with poor primaquine response (*33*).

Malaria cases increased during the early 1970s but were decreasing when Gabaldón retired in 1973 (Figure 3) (*28*). Gabaldón noted that cases were reintroduced by agricultural laborers into malaria-free regions where insecticides were not applied. This finding led to >100 new foci per year, often near malaria-endemic areas. The foci typically involved immunologically naive populations and were easy to identify by vigilance services. Applying DDT successfully to migratory Amerindian populations and behaviorally refractory mosquitoes was difficult. Venezuela reversed the increase in malaria incidence by the late 1970s (Figure 2, panel B; Figure 3). In 1983, Gabaldón claimed that his malaria control approach empowered his eradication success (*1*). Unfortunately, malaria incidence in Venezuela increased as the 1980s began.

Conclusions

The early success of malaria control in Venezuela was caused by interruption of malaria transmission through

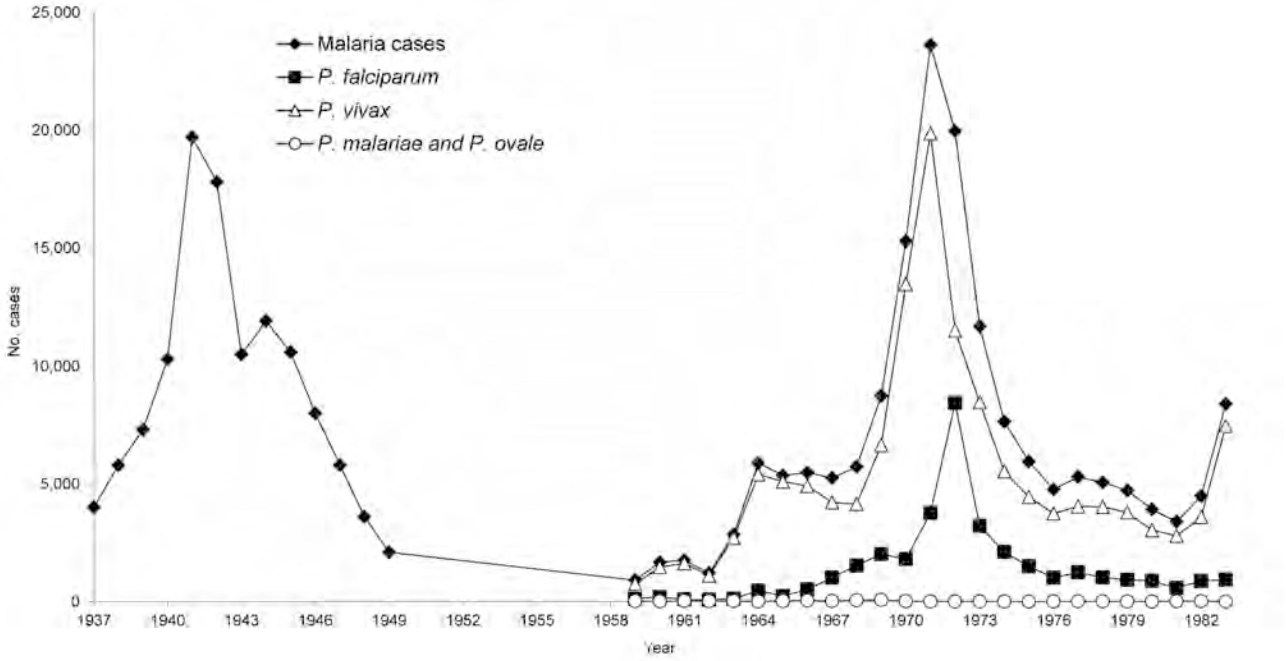

Figure 3. Annual malaria cases, by *Plasmodium* species, Venezuela, 1937–1983. Data for 1949 and earlier are estimates but remaining data are exact (*8*,*17*,*34*).

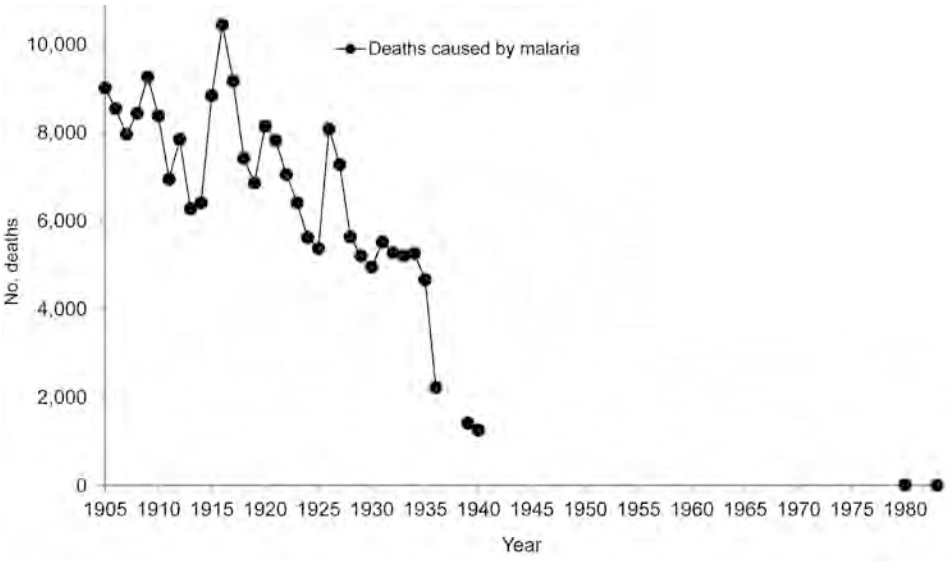

Figure 4. Malaria mortality rates, Venezuela, 1905–1983 (*35*,*36*). Arnoldo Gabaldón acknowledged that early malaria mortality rates in Venezuela had inherent limitations compared with rates for countries in temperate zones. The main limitations were deaths that were not registered or cases that were not diagnosed. These limitations were partially due to insufficient numbers of doctors covering the low-density populations and variations in data reporting between states and over time (*36*). Later estimates are likely of higher quality (*35*). Other mortality rates reported by Gabaldón and Berti are probably accurate because they are either generalizations or specific data that they likely considered accurate (*1*,*2*).

systematic and integrative infection and vector control. This control included detailed knowledge of malaria epidemiology at the local level (microepidemiology); case management (diagnosis, patient treatment, and mass drug administration); mapping malaria cases; a malaria health information system updated weekly; community participation through volunteer community health workers; application of larvicides and imagocides; and sanitary engineering (housing improvement, water management). Before DDT was available, Gabaldón used these tools to reduce malaria incidence by 40% during 1941–1944 and malaria-associated deaths by 45% during 1936–1940 (Figures 3, 4) (*17*). However, DDT was a key factor in the eradication program in Venezuela when it became available in 1945.

The approach of Gabaldón to malaria eradication differs little from modern day prevention, control, and elimination, although it was implemented in a world where vector and parasite resistance were distant rumbles and governmental support was strong. However, this approach diverged from later stages of malaria eradication defined by WHO. Gabaldón integrated malaria control with sanitary engineering, rather than with clinical treatment. He also acknowledged that in a world of porous borders, malaria reintroductions would continue. Therefore, vector control would require long-term investment.

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Haemophilus ducreyi Associated with Skin Ulcers among Children, Solomon Islands

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During a survey of yaws prevalence in the Solomon Islands, we collected samples from skin ulcers of 41 children. Using PCR, we identified *Haemophilus ducreyi* infection in 13 (32%) children. PCR-positive and PCR-negative ulcers were phenotypically indistinguishable. Emergence of *H. ducreyi* as a cause of nongenital ulcers may affect the World Health Organization's yaws eradication program.

Bacterial ulcerative skin diseases are a common cause of illness in the developing world (*1*). Some of these diseases, including Buruli ulcer, caused by *Mycobacterium ulcerans*, and yaws, caused by *Treponema pallidum* subspecies *pertenue*, occur only in tropical and subtropical climates. Yaws is endemic in the Solomon Islands, where ≈15,000 cases per year are reported (*2*). In 2012, the World Health Organization (WHO) launched a worldwide yaws eradication program based on treatment by mass distribution of azithromycin and monitoring for skin ulcers (*3*).

Reports suggest that *Haemophilus ducreyi*, the causative organism of chancroid, a sexually transmitted infection, may be associated with nonsexual transmission of nongenital ulcers of the skin in persons from the Pacific region (*4*,*5*). If this organism is a common cause of skin ulcers in the region, this factor has crucial implications for the yaws eradication strategy. PCR has been shown to be highly sensitive and specific for diagnosing chancroid (*6*). We used real-time PCR to detect *H. ducreyi* in skin ulcer samples collected during a survey for yaws in the Solomon Islands.

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The Study

We conducted a cross-sectional survey for yaws in the Western Province and Choiseul Province of the Solomon Islands in 2013. In each province, we chose 25 clusters using a probability-proportionate-to-size method. In each cluster, we selected 30 houses by random sampling; children 5 to 14 years of age living in those houses were invited to participate. Informed written consent was obtained from the children's parents.

Children underwent standardized examination. We recorded location, classification, and duration of skin lesions and yaws treatment history using the LINKS system (*7*, http://www.linkssystem.org/). Lesions were classified by using the WHO pictorial grading scheme for yaws (*8*). Tenderness was classified based on reports by children. Blood samples were collected from all children. For children with exudative skin lesions, a sample for PCR was collected by rolling a sterile cotton-tipped swab across the lesion and placing it in a cryotube pre-filled with 1.2 mL of AssayAssure solution (Thermo Fisher Scientific, Waltham, MA, USA). Samples were transferred to Honiara National Referral Hospital within 5 days and stored at –20°C. Serum samples were placed on dry ice and shipped to the London School of Hygiene & Tropical Medicine and lesion samples to the US Centers for Disease Control and Prevention.

Serum samples were tested by using *T. pallidum* particle agglutination (Mast Diagnostics, Merseyside, UK) at the London School of Hygiene & Tropical Medicine. For samples with a positive *T. pallidum* particle agglutination, a rapid plasma regain test was performed (Deben Diagnostics, Ipswich, UK). DNA was extracted from lesion samples in a CDC laboratory by using iPrep PureLink gDNA blood kits and the iPrep purification instrument (Life Technologies, Grand Island, NY, USA). A real-time duplex PCR targeting the DNA polymerase I gene (*polA*, *tp0105*) of pathogenic treponemes (which detects all 3 *T. pallidum* subspecies) and the human *RNase P* gene (to monitor for PCR inhibition) was performed by using a Rotor-Gene-Q real-time PCR instrument (QIAGEN Inc., Valencia, CA, USA) (*9*). Negative (no-template) control and positive controls for *T. pallidum* DNA were included in each PCR run. Considering reports of *H. ducreyi* and the occurrence of *M. ulcerans* in Papua New Guinea, immediately north of the Solomon Islands, we performed a second duplex real-time PCR for *M. ulcerans* and *H. ducreyi* on all samples by using previously validated targets (*10*,*11*).

For the purpose of analysis, lesions were classified as acute (<4 weeks) or chronic (>4 weeks). A rapid plasma regain titer ≥1/4 was considered positive. Fisher exact test was used to compare characteristics of patients whose lesions contained *H. ducreyi* with patients whose lesions did not contain *H. ducreyi*. Analyses were performed by using STATA 13.1 (http://www.stata.com/).

During the survey, 1,497 children were examined. Samples for PCR were collected from 41 children who had exudative lesions (19 male, median age 8 years). Twenty-two children had ulcerative lesions from which a sample could not be collected. Twelve (29.3%) children had positive results for yaws from serologic testing, but no DNA evidence of *T. pallidum* subsp. *pertenue* or *M. ulcerans*, causative organisms of yaws and Buruli ulcer, respectively, was detected in any sample. *H. ducreyi* DNA was amplified from 13 (32%) samples (Figure). PCR inhibitors were not found in any samples. Clinical data were incomplete for 2 participants. There were no notable differences in the recorded characteristics of skin lesions or in the serologic status of patients in whose ulcers *H ducreyi* DNA was found compared with those in which *H ducreyi* was not found (Table).

Conclusions

H. ducreyi is frequently present in skin ulcers of children in the Solomon Islands, and lesions containing *H ducreyi* DNA were similar in location, duration, and tenderness to lesions in which *H ducreyi* was not found. Papua New Guinea reported a similar finding (*12*). Experimental models of chancroid have demonstrated that injection of *H. ducreyi* into the epidermis and dermis causes nongenital skin disease, suggesting that *H. ducreyi* may be the cause of some ulcers in our survey (*13*). Similar to results for experimental models, *H. ducreyi* DNA was found more frequently in samples collected from boys (8/13; p = 0.179), although this difference was not statistically significant. It is possible that the difficulty of collecting samples for molecular testing, the lack of facilities to enable collection of samples for culture in affected areas, and the precise culture requirements of *H. ducreyi* have notably delayed recognition of this association (*14*).

Lesions associated with *H. ducreyi* were found in patients with positive and negative serologic test results for *T. pallidum* subsp. *pertenue*. It is likely that patients with positive serologic test results represent latent yaws with an alternative etiologic agent causing the current lesion. The possibility that there are alternative causes of childhood skin ulcers in the Pacific region could have implications for WHO's yaws eradication strategy, which is based on detection of suspected clinical cases. Although azithromycin is effective in treating genital strains of *H. ducreyi* and experimental nongenital lesions (*15*), further studies are needed to confirm efficacy in nongenital lesions in a clinical setting. The emerging data suggest that surveillance strategies should routinely require molecular diagnostics.

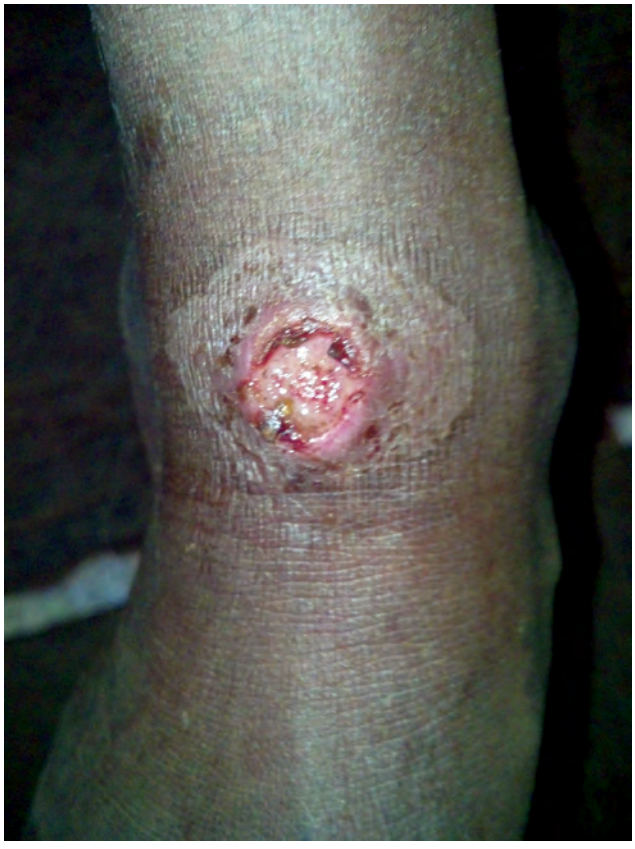

Figure: Example of lesion from which sample was obtained and *Haemophilus ducreyi* DNA was amplified, Solomon Islands, 2013. Photograph ©2014 Michael Marks.

A causative agent was not identified in a large proportion of lesion samples. A variety of possible reasons exist for this, including the fact that some lesions were noninfectious, such as insect bites, some numbers of organisms were below current limits of detection of real-time-PCR, or that other organisms, such as staphylococci, for which PCR was not performed, caused these lesions. The sample collection/transport media and PCR assays we used varied from those used by Mitjà et al (*12*), but it is unclear to what extent this effected our results. A single

Table. Comparison of skin ulcer samples from 41 patients tested for *Haemophilus ducreyi*, Solomon Islands, 2013*

Characteristic	No. (%) samples tested for *H. ducreyi* by real-time-PCR, 95% CI		p value
	Positive, n = 13	Negative, n = 28	
Male sex	8 (62), 32%–86%	10 (36), 19%–56%	0.179
Location of lesion on leg	12 (92), 64%–99%	21 (96), 80%–99%	0.561
Duration <4 weeks	7 (54), 25%–81%	14 (54), 33%–73%	0.632
Painful lesion	8 (62), 32%–86%	17 (65), 44%–83%	0.542
Sample TPPA-positive	6 (46), 19%–75%	17 (61), 41%–78%	0.503
Sample TPPA- and RPR-positive	5 (38), 14%–68%	7 (25), 11%–45%	0.469

*TPPA, *Treponema pallidum* particle agglutination; RPR, rapid plasma regain test.

sample was collected per patient, but several patients (n = 8, 19.5%) had >1 skin lesion. Swabbing every lesion may have increased the diagnostic yield for *H. ducreyi* and/or *T. pallidum* subsp. *pertenue*. Further studies to explore causes of skin ulcers in this community are needed to better inform disease control efforts.

Because it was not anticipated that *H. ducreyi* DNA would be found in nongenital skin lesions, we did not prospectively collect data on regional lymphadenopathy; however, we did not notice marked lymphadenopathy or bubo formation. Collection of samples for culture and sequencing of the *H. ducreyi* genome are needed to inform our understanding of relatedness of these strains to genital strains.

This study has 2 main limitations. First, the number of samples tested was small. Second, lesion samples were tested for only 3 organisms, raising the possibility that other organisms caused a large proportion of skin ulcers. Despite these limitations, this study clearly demonstrates that *H. ducreyi* is frequently present in childhood skin ulcers in this yaws-endemic community. Further studies of the epidemiology, microbiology, and response to treatment for this newly described pathogen–disease association are required.

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Dr Marks is a Wellcome Trust Clinical Research Fellow at the London School of Hygiene and Tropical Medicine. His research focuses on the impact of mass administration of azithromycin on treponemal infections as part of a strategy to eliminate yaws in the Solomon Islands.

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Human Granulocytic Anaplasmosis, South Korea, 2013

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We report a patient with human granulocytic anaplasmosis in South Korea. The patient had fever and thrombocytopenia. Human granulocytic anaplasmosis was confirmed by seroconversion, PCR, and sequence analysis for *Anaplasma phagocytophilum*. Morulae were observed in the cultured HL-60 cells inoculated with blood from the patient.

Anaplasma phagocytophilum, the causative agent of human granulocytic anaplasmosis (HGA), is a zoonotic tickborne pathogen transmitted by ixodid ticks that infects wild and domestic mammals and humans (*1*–*3*). HGA was first identified in the United States in 1994 (*1*) and subsequently in countries in Europe (*3*), China (*4*), and Japan (*5*).

To our knowledge, there is no report regarding the clinical description of HGA patients in South Korea. However, *A. phagocytophilum* has been detected in *Haemaphysalis longicornis*, *Ixodes nipponensis*, and *I. persulcatus* ticks (*6*,*7*) in this country. Molecular epidemiologic studies detected *A. phagocytophilum* in 2.6% (5/196) of striped field mice (*7*,*8*) and in 63.6% (42/66) of Korean water deer (*9*). Seroprevalence studies showed that 1.8% of serum samples from patients with acute fever were positive for *A. phagocytophilum* by an immunofluorescence antibody test (*10*). We report a patient with HGA and characterized the *A. phagocytophilum* isolate from this patient.

The Study

On May 17, 2013 (day 0, day of illness onset), fever, chills, nausea, and vomiting developed in a 57-year-old woman who lived in Chuncheon, Gangwon Province, South Korea. She came to a local clinic on the same day, and treatment with antipyretic drugs was initiated. The patient reported being bitten by a tick on her right shoulder while mountain climbing in Gangwon Province 5 days before the fever occurred. She did not recall having contact with any domestic animals and had no history of travel outside South Korea in the month before illness onset.

On day 4, the patient came to Kangwon National University Hospital (Chuncheon, South Korea) with a persistent fever despite use of antipyretics. The patient had a temperature of 39.2°C, blood pressure of 76/61 mm Hg, heart rate of 88 beats/min, and an oxygen saturation level of 98.3% on room air. An erythematous maculopapular lesion, 3 cm in diameter, was observed around the tick bite on the right shoulder. Laboratory tests showed pancytopenia and increased serum aminotransferase levels (Table).

On day 5, the patient was treated with norepinephrine, ceftriaxone, and doxycycline. On day 7, her blood pressure and temperature were within reference ranges and a norepinephrine infusion was discontinued. Peripheral blood smear showed no evidence of malaria, and antibody titer against *Orientia tsutsugamushi* was <1:40. Test results for antibodies against Hantaan virus, leptospira, *Borrelia burgdorferi*, and *Coxiella burnetii* were negative. Blood cultures prepared at the time of the visit were negative. On day 11, she was discharged from the hospital, and treatment with antimicrobial drugs was discontinued.

Because symptoms and laboratory findings, such as thrombocytopenia, anemia, and increased aminotransferase levels (Table), of the patient were similar to those for severe fever with thrombocytopenia syndrome (SFTS), anticoagulated blood and serum were obtained on day 5 before treatment with doxycycline and on day 19. Samples were sent to Seoul National University College of Medicine for additional diagnostic tests.

RNA was extracted from blood by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Reverse transcription PCR for SFTS virus was performed according to described methods (*11*) and showed negative results. No cytopathic effect was observed in Vero cells and DH82 cells after inoculating them with a blood sample.

DNA was extracted from the blood by using the QIAamp DNA Mini Kit (QIAGEN) to detect *A. phagocytophilum* 16S rRNA gene, *ankA*, *groESL* operon, and *msp2*. Nested PCR was conducted to amplify a 926-bp fragment of the 16S rRNA gene by using an *A. phagocytophilum* species-specific primer set (*12*). Direct sequencing of the PCR product was performed to confirm *A. phagocytophilum*, and the partial 16S rRNA gene sequence was deposited in GenBank (accession no. KF805344).

A nucleotide basic local alignment search tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) with the obtained sequence showed matches to only *A. phagocytophilum* sequences (99.3%–100% similarities). Aligning ≥2 sequences by using BLAST showed that the obtained sequence

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Table. Laboratory findings for patient with human granulocytic anaplasmosis, South Korea, 2013*

Laboratory test (reference range)	Day 5†	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 19‡
Leukocytes, cells/μL (3,800–10,000)	2,400	1,100	7,400	5,800	5,200	5,700	4,800	5,700
Neutrophils, % (40–70)	91	28	79	62	50	42	39	51
Lymphocytes, % (20–50)	7	64	17	31	39	45	47	42
Hb, g/dL (12.3–15.3)	9.4	9.3	9.4	8.7	8.8	10.4	9.8	10.3
Hct, % (36.6–44.2)	28.5	27.3	28.8	26.4	27.4	32.5	30.5	31.0
Platelets/μL (140,000–400,000)	78,000	61,000	63,000	69,000	105,000	194,000	249,000	348,000
AST, IU/L (15–41)	137	179	80	48	106	111	64	25
ALT, IU/L (14–54)	60	93	73	55	84	105	83	26
CPK, IU/L (38–234)	74	156	148	64	41	31	NA	39
LDH, IU/L (100–190)	280	384	275	203	243	239	180	134
Creatinine, mg/dL (0.4–1.0)	0.8	0.7	0.5	0.5	0.5	0.7	NA	0.6
C-reactive protein, mg/dL (0–0.50)	6.11	7.04	NA	NA	NA	NA	0.62	NA
PT, INR (0.92–1.17)	1.05	1.17	1.00	0.97	NA	NA	NA	NA
aPTT, s (31.0–43.7)	44.8	57.6	49.3	41.5	NA	NA	NA	NA
Fibrinogen, mg/dL (207–408)	NA	304	328	308	NA	NA	NA	NA

*Day 0 was day of illness onset. Hb, hemoglobin; Hct, hematocrit; AST, aspartate aminotransferase; ALT, alanine aminotransferase; CPK, creatine phosphokinase; NA, not available; LDH, lactate dehydrogenase; PT, prothrombin time; INR, international normalized ratio; aPTT, activated partial thromboplastin time.
†Blood samples for culture and for immunofluorescence antibody test were obtained.
‡Blood samples for immunofluorescence antibody test were obtained.

had lower similarities (94.3%–98.6%) with other *Anaplasma* species (*A. marginale* [GenBank accession no. NR_074556], *A. centrale* [NR_074356], *A. ovis* [AY262124], *A. platys* [EF139459], and *A. bovis* [HM131218]) and with *Ehrlichia* species (*E. chaffeensis* [NR_037059], *E. ewingii* [NR_044747], and *E. canis* [NR_074386]).

Comparison of the 16S rRNA gene sequence with sequences for other *A. phagocytophilum* strains from South Korea showed that this sequence was relatively distant from those of Korean water deer isolates, but similar to those of tick isolates from Jeju Island (Figure 1, panel A). Comparison of the 16S rRNA gene sequence with other sequences

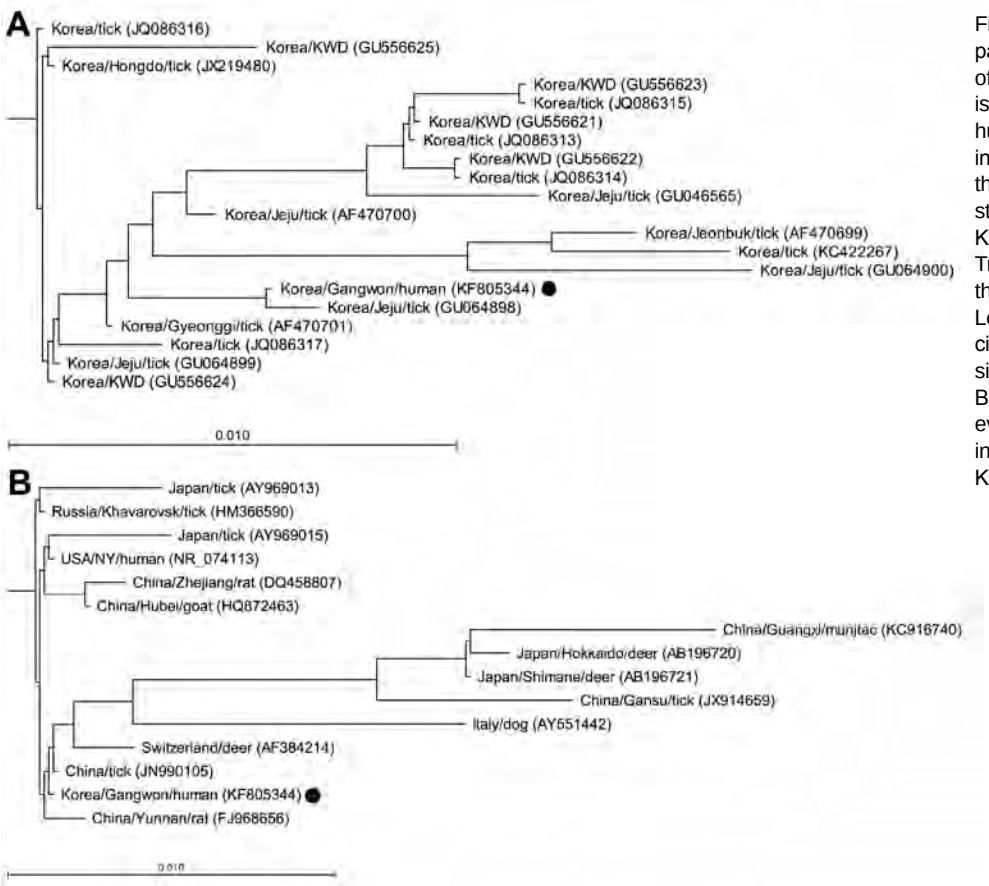

Figure 1. Phylogenetic trees for partial 16S rRNA gene sequences of an *Anaplasma phagocytophilum* isolate obtained from a patient with human granulocytic anaplasmosis in South Korea (black dots) and those of the *A. phagocytophilum* strains reported from A) South Korea and B) other countries. Trees were constructed by using the neighbor-joining method. Locations (country/province or city), hosts, and GenBank accession numbers are indicated. Branch lengths of trees show evolutionary distances. Scale bars indicate 1.0% sequence distance. KWD, Korean water deer.

of *A. phagocytophilum* strains reported from other countries showed that our isolate was relatively distant from the strains obtained from ticks collected in Gansu and Guangxi, China, and Hokkaido and Shimane, Japan. The isolate was similar to a human isolate from the United States, tick isolates from Russia and China, and animal isolates from Yunnan, Zhejiang, and Hubei, China (Figure 1, panel B).

Nested PCR was performed to amplify a 667-bp fragment of the *ankA* gene (*13*), and single non-nested PCRs were performed to amplify a 1,715-bp fragment of the *groESL* operon (*14*) and a 334-bp fragment of the *msp2* gene (*15*). Direct sequencing of PCR products was performed to confirm *A. phagocytophilum*, and partial *ankA*, *groESL*, and *msp2* sequences were deposited in GenBank (accession nos. KJ677106–KJ677108). Nucleotide BLAST searches with obtained sequences showed matches to only *A. phagocytophilum* sequences: 94.1%–97.9% similarities for *ankA*, 97.9%–99.5% for *groESL*, and 97.8%–99.6% for *msp2*.

For *A. phagocytophilum* culturing, a suspension of human promyelocytic cell line HL-60 (ATCC CCL-240) was inoculated with the patient's blood sample (day 5 postillness, before treatment with antimicrobial drugs) and incubated at 37°C in an atmosphere of 5% CO_2 (*10*). The culture suspension was examined microscopically by Wright-Giemsa staining of cytocentrifuged preparations of cells at 2 to 3-day intervals. Subculture was performed on day 14 postinoculation. On day 8 post-subculture, morulae within cultured HL-60 cells were observed (Figure 2).

Nested PCRs to amplify 16S rRNA and *ankA* genes showed positive results for isolates from day 14 postinoculation and from day 10 postsubculture. Single, non-nested PCRs to amplify *groESL* and *msp2* genes showed positive results for the isolate from day 14 postinoculation but negative for the isolate from day 10 postsubculture. Comparison of sequences from culture isolates and those from PCR products directly amplified from the blood sample showed that these sequences were identical.

For serologic diagnosis, an immunofluorescence antibody test kit for *A. phagocytophilum* (Fuller Laboratories, Fullerton, CA, USA) was used. Positive cutoff titers were 1:16 for IgM and 1:80 for IgG, according to the manufacturer's instructions. Specific IgM titers increased from 1:16 (day 5) to ≥1:256 (day 19). Specific IgG antibody titers increased from 1:20 (day 5) to 1:160 (day 19).

Conclusions

We confirmed a case of HGA in a patient in South Korea (who was suspected of having SFTS) by using serologic analysis, PCR, culture, and sequence analysis for *A. phagocytophilum*. According to the case definition of HGA of the US Centers for Disease Control and Prevention (Atlanta, GA, USA) (*2*), this case fulfilled the criteria for anaplasmosis. The patient had a history of a tick bite, and the clinical symptoms and laboratory findings were similar to those of SFTS, an emerging vector-borne disease in South Korea (*11*).

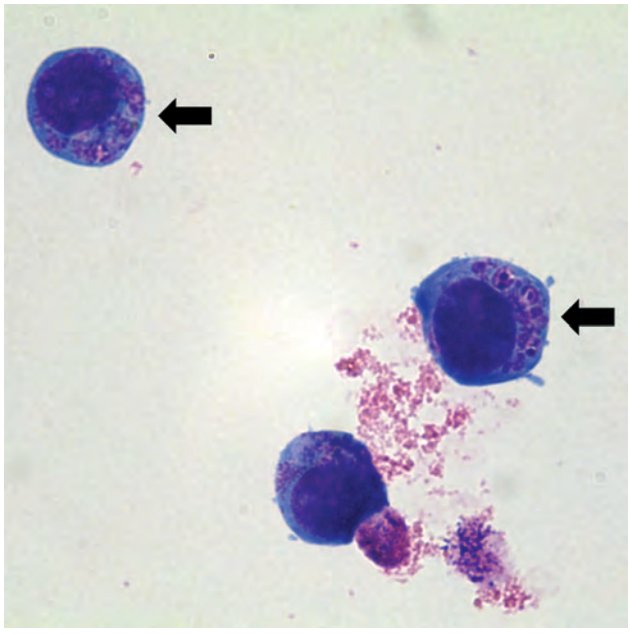

Figure 2. Light micrograph of *Anaplasma phagocytophilum* cultured in human promyelocytic cell line HL-60, showing *A. phagocytophilum* morulae as basophilic and intracytoplasmic inclusions (arrows). Wright–Giemsa stain, original magnification ×1,000.

No effective treatment for SFTS is available, but doxycycline is effective for treating HGA (*2*). Clinical features of HGA may be confused with those for SFTS, which may result in inappropriate treatment and severe outcomes. Therefore, not only SFTS but also HGA should be considered as differential diagnoses for patients with fevers and thrombocytopenia after tick bites.

Dr Kye-Hyung Kim is an infectious disease physician and a senior researcher at Seoul National University College of Medicine, Seoul, South Korea. Her primary research interests are medical virology and emerging infectious diseases.

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Ongoing Epidemic of Cutaneous Leishmaniasis among Syrian Refugees, Lebanon[1]

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In September 2012, a cutaneous leishmaniasis outbreak began among Syrian refugees in Lebanon. For 948 patients in whom leishmaniasis was not confirmed, we obtained samples for microscopic confirmation and molecular speciation. We identified *Leishmania tropica* in 85% and *L. major* in 15% of patients. After 3 months of megulamine antimonite therapy, patients initial cure rate was 82%.

The Syrian population has been affected by the protracted conflict and ongoing insecurity in the Middle East. The United Nations High Commissioner for Refugees estimates that >4.1 million refugees have been displaced into temporary settlements in neighboring countries, including 1.5 million into Lebanon (*1*,*2*). Persons in these temporary settlements are affected by inadequate sanitation, lack of access to clean water, overcrowding, and increased exposure to disease.

Leishmaniasis is a parasitic disease comprising a wide spectrum of chronic infections in humans and in certain animal species. It is caused by ≈20 species of *Leishmania* protozoa; is distributed worldwide; and affects millions of persons in parts of Asia, Africa, South America, and the Mediterranean Basin. The global incidence is ≈1.5–2 million cases per year, and the disease primarily affects children (*3*). Leishmaniasis is a major public health concern in the eastern Mediterranean region, which bears the brunt of the worldwide prevalence (≈57%), and is endemic to 16 of the 23 countries in this region, and Afghanistan, Iraq, and the Syrian Arab Republic are among the hot spots of leishmaniasis (*4*).

The 3 major forms of leishmaniasis (cutaneous, mucocutaneous, and visceral) are transmitted by the bite of the female sandfly (*5*). *L. major*, the most widely endemic disease-causing species, is closely tied to the semiarid climate zone (*6*). *L. tropica* is found in countries in northern Africa, central Asia, and the Middle East, including northern Syria (Aleppo) (*7*). Aleppo is one of the most cutaneous leishmaniasis–endemic areas in the world; ≈12,000 new cases occur each year (*8*). We have documented a cutaneous leishmaniasis outbreak among Syrian refugees within the Lebanese borders that began in September 2012 and is ongoing.

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The Study

The institutional review board of the American University of Beirut approved this study. In November 2012, one of us (I.K.) began documenting the epidemic during trips to multiple refugee camps in eastern Lebanon (the Bekaa Valley [Baalbek, Zahle, and Ersaal]); the epidemic later expanded to camps throughout the country. During the study, 1,275 patients from 213 displaced families were triaged into 3 groups: l) leishmaniasis diagnosed, confirmed, and partially treated; 2) leishmaniasis diagnosed, confirmed, but not treated; and 3) leishmaniasis not diagnosed. The first 2 groups comprised 55 families; the remaining 158 families (948 persons) were triaged for diagnosis confirmation and are included in the subsequent statistical analyses. Data were collected at the refugee camps and included punch biopsy specimens for 1 patient/family. We used punch biopsy for microscopic confirmation and for molecular analysis by PCR using a previously published protocol (*9*). The anatomic sites for biopsy were selected on the basis of appropriate recommendations for sampling (*10*).

The average age of patients was 17.6 years; most (80%) patients were <18 years of age. Each family comprised 3–13 members (mean 6), and the percentage of family members infected ranged from 8% to 100% (mean 52%). The refugees had fled from areas to which leishmaniasis is endemic and nonendemic. Most of the refugees we encountered had migrated from Aleppo (74 [67.3%] patients), followed by Homos (30 [27.3%] patients) and Damascus (6 [5.4%] patients). Only 1 Lebanese resident with no history of travel during the past 5 years had leishmaniasis. Our sample population was scattered predominately among 4 regions of Lebanon (Figure 1).

The refugees in our study had been in Lebanon for 1–24 months (mean 5 months) and reported that the first time they saw a cutaneous lesions was 1–27 months (mean 5 months) before being examined. Most (77%) patients reported the appearance of the first lesion after being in Lebanon for >2 months, and 53% of patients recalled history of

[1]This research was presented at the 103rd Annual Meeting of the United States and Canadian Academy of Pathology, March 1–7, 2014, San Diego, California, USA.

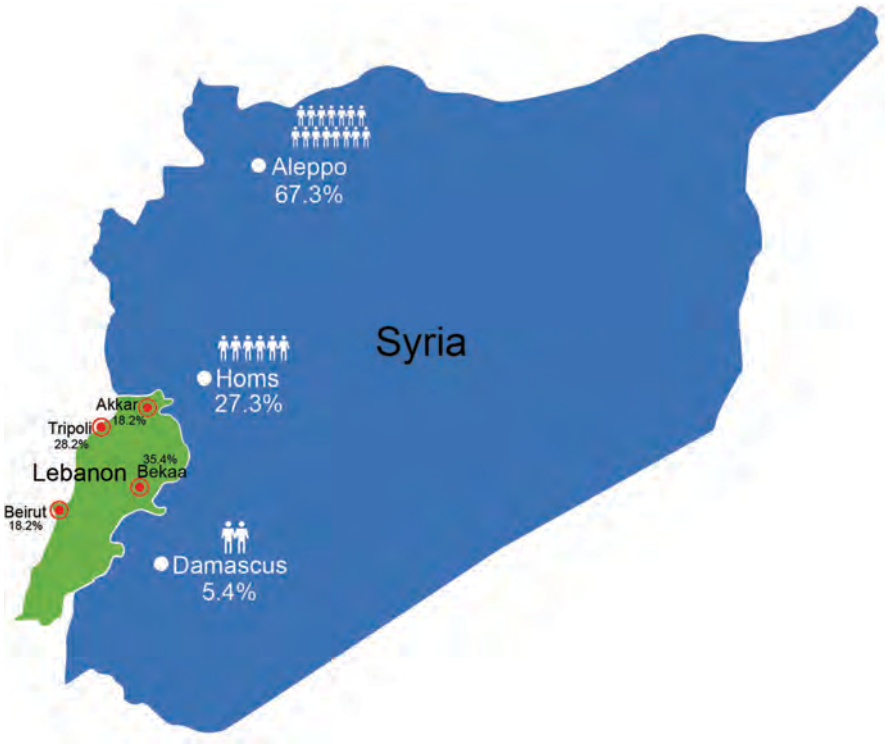

Figure 1. Migration patterns of refugees with cutaneous leishmaniasis identified in Lebanon starting in late 2012, showing movement from cities in Syria (white dots) to regions in Lebanon (red dots). Human figures and percentages shown in Syria indicate relative proportions of refugees to Lebanon from each city in Syria; most (67.3%) patients had migrated from Aleppo, where leishmaniasis is endemic. Percentages shown in Lebanon indicate percentages of refugees who had migrated to each location.

This research was presented at the 103rd Annual Meeting of the United States and Canadian Academy of Pathology, March 1–7, 2014, San Diego, California, USA. an antecedent insect bite. The head and neck were the most common locations for the cutaneous lesion (43% of patients), followed by the upper extremities (26%) and lower extremities (11%). Patients had 1–15 lesions (mean 3). Lesion size ranged from 1 cm to 15 cm (average 2.6 cm). Verrucous lesions, with or without ulceration, were the most common lesion type (56%), followed by plaque/nodular lesions (43%) and papular lesions (2%). Most patients (83%) had dry lesions; 7 persons (4%) had primary wet lesions, and 12 (8%) had both. All patients had active lesions without evidence of healing or scarring. Speciation by PCR yielded *L. tropica* in 85% of patients and *L. major* in 15%.

Fifty-nine percent of patients had ≥1 of the following: disease compromising the function of vital sensory organs (eye, ear, nose, and mouth) (27%); lesions >5 cm in diameter (49%); disfiguring facial lesions (37%); special forms, such as sporotrichoid or lymphangietic with satellite lesions (9%); and lesions present for >12 months' duration (9%) (Figure 2). The above parameters were more prevalent among children (age range 3 months–16 years; median 9 years vs. 21 years; $p = 0.002$) and more frequently observed on the face and lower extremities ($p = 0.002$).

In May 2013, the World Health Organization (WHO) donated 10,000 doses of megulamine antimonate (85 mg/mL; 5-mL ampoules). As of April 2013, 5,091 ampoules of intralesional or intramuscular medication had been used to initiate or continue treatment of all 1,275 patients triaged. Eighty percent received intralesional therapy, and 20% received intramuscular therapy in accordance with WHO recommendations (*4*).

Systemic therapy can have serious side effects. To provide adequate and humane medical care to this impoverished population, healthcare personnel administered local anesthetic with the intramuscular injections and conducted complete blood counts, liver function tests, analysis of creatinine levels, and electrocardiograms every 2 weeks for all patients on systemic therapy. No complications occurred.

Conclusions

Our data are based on the analysis of leishmaniasis in Syrian refugees in camps in rural areas of Lebanon. The camps were mainly makeshift houses of rubble and tents, plagued by inadequate sanitation, waste disposal, and insulation (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/10/14-0288-Techapp1.pdf). Poverty, malnutrition, population displacement, weakened immunity, and poor housing are all risk factors for cutaneous leishmaniasis (*11*). Such conditions are ideal for vectors of *L. tropica* and enable leishmaniasis to flourish as an anthroponotic disease, as seen in outbreaks in Kabul, Afghanistan (*12*).

Although Lebanon remains hypoendemic for cutaneous leishmaniasis, experience has suggested that rates within Lebanon were previously low and restricted to areas

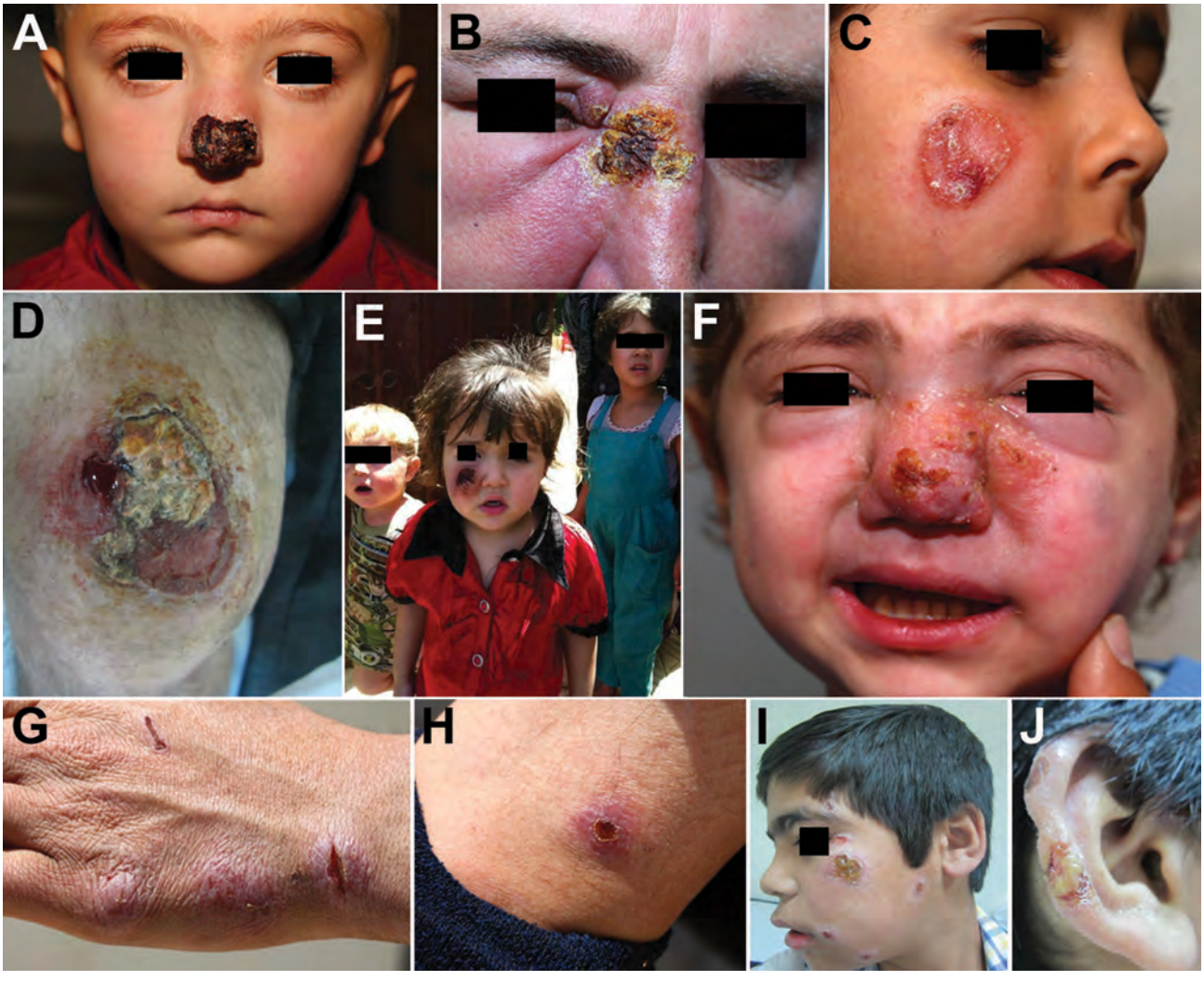

Figure 2. Patterns of leishmaniasis among Syrian refugees in Lebanon, 2012. A, B) Lesions impinging and possibly hindering the function of vital sensory organs, including the nose and eyes. C, D) Lesions >5 cm. E, F) Lesions disfiguring the face. G, H) Special forms of cutaneous leishmaniasis; shown here is a patient with spread and satellite lesions on the hand and arm. I, J) Patient with 15 lesions.

bordering Syria (*13*). The WHO leishmaniasis control team reported no cases in Lebanon during 2004–2008, compared with 22,882 cases in Syria during the same period (*14*). Furthermore, 85% of cases studied were caused by *L. tropica*, a species endemic to the Aleppo region in Syria. This finding might explain why most of the patients we encountered had aggressive (large, multiple, and disfiguring facial lesions) and prolonged disease courses necessitating treatment with intramuscular rather than intralesional medication. Local collaboration, early detection, and diagnosis, along with speciation of the parasite, were of paramount importance to ensure the effective delivery of treatment and successful control of the epidemic.

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etymologia

Knemidocoptic mange [ne- mĭ-do-kop′ tik mānj]

From the Latin *manducare* (to itch), mange is a skin disease caused by mites in domestic and wild animals. Knemidocoptic, from the Greek *knemid* (greave, a piece of armor that protects the leg) and *koptein* (to cut), refers to the morphology and pathogenesis of mites of the genus *Knemidocoptes*, which are burrowing mites of birds. Commonly known as scaly face, scaly legs, or tassel foot, knemidocoptiasis affects primarily the face and legs of birds around the world worldwide and can be fatal.

—Begaleaon Helene Somda

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Knemidocoptic Mange in Wild Golden Eagles, California, USA

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During 2012–2013 in California, USA, 3 wild golden eagles were found with severe skin disease; 2 died. The cause was a rare mite, most closely related to *Knemidocoptes derooi* mites. Cautionary monitoring of eagle populations, habitats, and diseases is warranted.

Knemidocoptidae are mange mites that cause alopecia, acanthosis, hyperkeratosis, pruritus, feather damage, inappetence, and sometimes death in birds (*1*,*2*). Infestation is typically worse in older, injured, sick, stressed, or malnourished birds (*3*). Most reports describe *Knemidocoptes mutans* and *K. gallinae* mites as affecting gallinaceous birds and *K. pilae* and *K. jamaicensis* mites as affecting pet or exotic birds in captivity (*1*,*2*). Although rarely fatal, epizootics among wild birds have been described; most affected raptors have been in captivity or rehabilitation centers (*2*,*4*,*5*). We describe the pathology caused by a rare species of mange mite in 1 of 3 wild golden eagles (*Aquila chrysaetos*) during an outbreak of knemidocoptosis.

The Cases

During December 2012, eagle 1 was found in Hollister, California, after being struck by a car. The 3.5-kg bird was admitted to the SPCA for Monterey County, Salinas, California; it was prostrate from head trauma and died overnight. Examination revealed feather loss and scabbing on the head, neck, legs, and near the cloaca; microscopic examination of a skin scraping revealed mites.

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During July 2013, eagle 2 was live-trapped in the Altamont Pass Wind Resource Area near Livermore, California. This subadult (*6*) female had skin lesions similar to those of eagle 1. After mites were detected, she received treatment at the Veterinary Medical Teaching Hospital, University of California, Davis, and underwent rehabilitation at the California Raptor Center. Skin scrapings with crusts were collected and stored at –20°C.

During August 2013, eagle 3 was found grounded in King City, California, and admitted to the SPCA for Monterey County. This 2.7-kg subadult male exhibited severe dehydration, weak and raspy respiration, and poor quality feathers. Mites and lesions similar to those on eagles 1 and 2 were found. Because of a poor prognosis, eagle 3 was euthanized and the carcass was stored at –20°C.

On August 15, 2013, a necropsy of eagle 3 was performed at the California Animal Health and Food Safety Laboratory System, Davis, California. The bird was thin and the skin of the head, neck, vent region, and legs (except feet) had rough, firm, thick, dry, scaly, gray-brown crusts (Figure 1, panel A). The skin thickness was up to 1 cm; the left eye and both ears were completely encrusted (Figure 1, panel B). Stereoscopy revealed white-to-transparent miliary bosselations beneath and within severely thickened keratin and a few crawling mites (Figure 1, panel C). The liver had disseminated pinpoint pale foci; lungs were heavy, wet, and dark red. Ancillary tests included aerobic bacterial culture of liver, spleen, and lung tissue and real-time PCR for *Salmonella* spp. in intestinal contents, for avian influenza virus and avian paramyxovirus-1 on an oropharyngeal swab sample, and for West Nile virus in kidney tissue. The femur was screened for chronic lead exposure, and liver tissue was screened for selenium, lead, manganese, cadmium, copper, iron, zinc, molybdenum, arsenic, mercury, and anticoagulant rodenticides. Bacteria and viruses were not detected, and all toxicants were either not detected or within acceptable range (except 2.1 ppm lead in bone and 0.01 ppm brodifacoum in liver).

Organ samples were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Histopathologic examination revealed generalized loss of feather follicles and numerous intracorneal mites. The epidermis was diffusely scalloped and covered by dense orthokeratotic lamellae, which formed serially aligned pouches within the stratum corneum; pouches contained developing and adult mites and eggs, mostly stacked and forming a honeycomb appearance. Subjacent epidermis was atrophied; acanthotic septae and papillary projections were overlain by orthokeratotic spires separating pouches. Coccoid bacteria and plant fragments were often associated with crusts. Throughout the dermis, small perivascular to interstitial heterophilic and pleocellular infiltrates were present. Acute necrotizing foci stippled with karyorrhexis

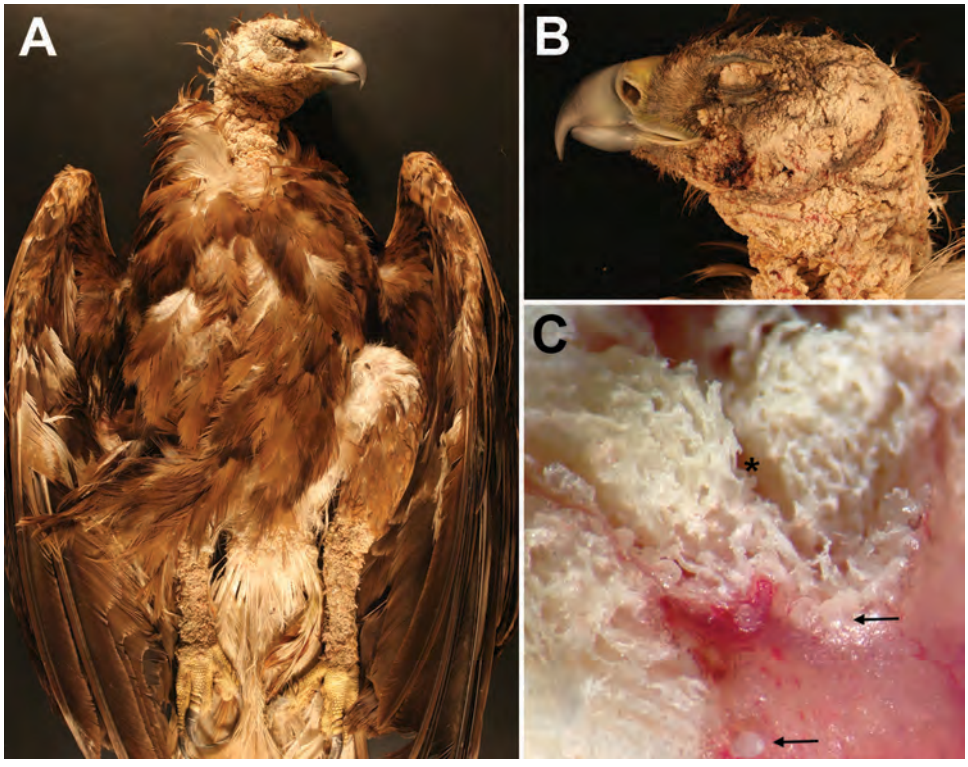

Figure 1. Golden eagle found grounded in King City, California, USA, during August 2013 (eagle 3). A) Photograph of diffuse crusting and thickening of the head, neck, and legs. B) Photograph showing severe obliteration of the skin over the eyelid and ear. C) Dissecting microscopic cross-section of the affected skin, showing thick trabeculae of keratin deposition (*) and white to transparent mites (arrows). Original magnification ×.

and fibrin exudates and small plasmacytic infiltrates were found in myocardium, peripheral nerves and perineural tissues, liver, spleen, and lungs. Abundant protozoa were seen in lungs and heart; intense immunoreactivity to *Sarcocystis falcatula* protozoa was found in vessels, lungs, heart, and perineural tissues; occasional immunoreactivity was found in dermis and brain endothelium.

Frozen skin specimens from eagles 2 and 3 were submitted for parasite identification by electron microscopy and PCR. Mites were fixed with Karnovsky fixative in 0.1 mol/L Sorenson sodium phosphate buffer, washed with sodium phosphate, dehydrated in increasing ethanol to 100%, and dried in a Tousimis Research Corporation 931.GL Autosamdri Critical Point dryer (Rockville, MD, USA). The mites were mounted on aluminum stubs, sputter-coated with gold, and viewed on an FEI XL30 TMP scanning electron microscope (Eindhoven, the Netherlands). The mites closely resembled *K. derooi* mites on the basis of the dorsal striation pattern, relative size of the propodosoma, dorsoterminal location of the anus, and dorsal positioning of the anterior legs and ventral positioning of the posterior legs (Figure 2) (*7*).

DNA was extracted from 2 pools of 5–10 mites from eagles 2 and 3 by using a QIAGEN Tissue Kit (Valencia, CA, USA). Modified PCR amplification (*8*) of a fragment of the *cytochrome oxidase* subunit I gene included 12.5 mL of GoTaq Green Master Mix (Promega, Madison, WI, USA), 1.0 mol/L of each primer, 2.5 mL of water, and 5 mL of DNA. Bands of 715 bp were cleaned with a QIAGEN gel extraction kit before DNA sequencing (Davis Sequencing, Davis, CA, USA). A BLAST search was conducted to match the DNA sequences with those in GenBank. Sequence homology was 100% between the pools from both eagles and 88% to *K. jamaicensis* (JQ037816.1), the only *Knemidocoptes* mite in the database. The GenBank accession number for the golden eagle mites is KJ787640.

Conclusions

The severity and diffuse distribution of skin lesions of these eagles suggest a possible serious, unique outbreak. Most knemidocoptic mites affect the face and cere, or legs and feet (called scaly face and scaly leg mites) (*1*,*2*). With such severe feather loss and crusting over the eyes and ears, irritation and limited thermoregulation probably decreased the ability of these eagles to feed and exacerbated clinical disease. The morphologic features of the mites were consistent with those of *K. derooi* mites, a species rarely reported and, to our knowledge, not reported in North America or on raptors (*7*). Although raptors sometimes have skin mites, such debilitating disease in otherwise healthy animals is highly atypical (*1*–*3*). Disease or exposure to contaminants might induce immunosuppression or toxicosis. Eagle 3 showed evidence of prior exposure to lead; although use of lead bullets has been banned in some areas of California since 2008, ingestion of spent lead ammunition in hunter-killed carcasses or viscera remains a

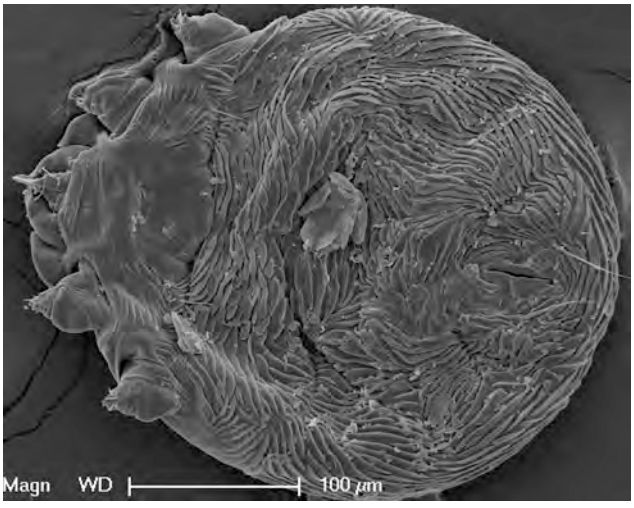

Figure 2. Electron microscopic image of mite, showing features consistent with *Knemidocoptes derooi* mites.

threat to wildlife throughout much of the western United States (*9*). Brodifacoum, a second-generation anticoagulant rodenticide also identified in this eagle, has been found in golden eagles (*10*) and could induce sublethal effects and immunosuppression. The respiratory disease probably resulted from *S. falcatula* infection, which can produce fatal systemic disease in wild eagles (*11*).

The effects of environmental and climate change on bird–mite relationships might be a factor in the emergence of mange. Because *Knemidocoptes* spp. mites are transmitted by contact, stress induced by crowding, toxicosis, and concurrent pathogens might account for the severe disease among birds in close proximity, or these cases might reflect the emergence of a highly virulent mite (*2*,*3*). The density of golden eagles near the Altamont Pass Wind Resource Area is notably high (*12*), and this outbreak might reflect habitat changes bringing individuals into closer contact or increasing stress. Consistent multidisciplinary data collection is needed for identification of the mite's host species range, ecology, and pathogenicity and for enhanced understanding of this possibly emerging fatal disease.

Acknowledgments

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Dr Mete is an assistant professor of veterinary pathology at the California Animal Health and Food Safety Laboratory, University of California, Davis. Her interests are spontaneous diseases with emphasis on avian pathology, and her research areas are iron storage disease in captive birds and diseases of backyard chickens.

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Novel Influenza A(H7N2) Virus in Chickens, Jilin Province, China, 2014

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In February 2014, while investigating the source of a human infection with influenza A(H7N9) virus in northern China, we isolated subtypes H7N2 and H9N2 viruses from chickens on the patient's farm. Sequence analysis revealed that the H7N2 virus is a novel reassortant of H7N9 and H9N2 viruses. Continued surveillance is needed.

Influenza subtype H7 viruses have been detected in poultry worldwide; associated human disease ranges from mild to severe (*1*–*8*). Human infections with influenza A(H7N9) viruses were first identified in China in March 2013 (*9*). As of March 11, 2014, a total of 375 laboratory-confirmed cases of human infection with influenza A(H7N9) virus, including 115 deaths, had been reported to the World Health Organization (*10*).

On February 21, 2014, the National Health and Family Planning Commission of China notified the World Health Organization of a laboratory-confirmed case of human infection with influenza virus subtype H7N9 (*11*). The patient was a 50-year-old farmer who lived in Jilin Province and traded chickens for a living. He became ill on February 15 and was confirmed to be infected with H7N9 virus on February 21. He recovered 2 weeks later. Although H7N9 viruses had been detected in live poultry markets in 12 provinces in China (*12*,*13*), the virus had not been detected in Jilin Province, in poultry or humans. To locate the origin of the infection, we conducted influenza virus surveillance among poultry in the patient's village.

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The Study

Cloacal and tracheal swab samples and serum were collected from 60 of 500 chickens on the patient's family farm and from 50 chickens in the backyards of 5 neighbors within 3 kilometers. In addition, 36 fecal samples from chickens on the patient's and neighboring farms were also collected. Each swab or fecal sample was placed in 2 mL of minimal essential medium supplemented with penicillin (2,000 U/mL) and streptomycin (2,000 U/mL). Virus was isolated by using 10-day-old specific pathogen–free embryonated chicken eggs. Hemagglutinin (HA) and neuraminidase (NA) subtypes were determined as described previously (*14*). Hemagglutination inhibition assay with 0.5% chicken erythrocytes was used to test for antibodies against H7 virus in the chicken serum samples.

From the cloacal swab samples from the patient's farm, 3 viruses were isolated (a Newcastle disease virus, an H9N2 influenza virus, and an H7N2 influenza virus); virus was not isolated from any sample collected from chickens on the neighboring farms. We designated the influenza viruses as A/chicken/Jilin/SD001/2014(H9N2) and A/chicken/Jilin/SD020/2014(H7N2).

We then fully sequenced the genomes of A/chicken/Jilin/SD020/2014(H7N2) and A/chicken/Jilin/SD001/2014 (H9N2) (GenBank accession nos. KM054788–KM054803) and found that the NA and nonstructural (NS) genes of A/chicken/Jilin/SD020/2014(H7N2) are similar to those of A/chicken/Jilin/SD001/2014(H9N2); identities were 99.1% and 100%, respectively. The other 6 genes were closely related to those of the H7N9 viruses that had been isolated from poultry or humans during 2013–2014 in China; identities were 99.5%–99.9% (Table). In the phylogenetic trees, the HA of A/chicken/Jilin/SD020/2014(H7N2) clustered with that of the recently emerged H7N9 viruses (online Technical Appendix Figure, panel A, http://wwwnc.cdc.gov/EID/article/20/10/14-0869-Techapp1.pdf), whereas, the NA, polymerase basic (PB) 2, PB1, polymerase acidic (PA), nucleocapsid protein (NP), and NS genes of A/chicken/Jilin/SD020/2014(H7N2) and A/chicken/Jilin/SD001/2014(H9N2) clustered with those of the H9N2 viruses (online Technical Appendix Figure, panels B–F, H). However, the matrix (M) gene of the 2 viruses remained on different forks; the M gene of A/chicken/Jilin/SD020/2014(H7N2) clustered with the H7N9 or H9N2 viruses, and the M gene of A/chicken/Jilin/SD001/2014(H9N2) clustered with the viruses from other subtypes (online Technical Appendix Figure, panel G). These results indicate that A/chicken/Jilin/SD020/2014(H7N2) is a novel reassortant of H7N9 and H9N2 viruses. With the approval of the Review Board of Harbin Veterinary Research Institute, we tested the virulence of the A/chicken/Jilin/SD020/2014(H7N2) in animals in Biosafety Level 3 laboratories.

Table. Homology among influenza viruses closely related to avian influenza virus A/chicken/Jilin/SD020/2014(H7N2) from Jilin, China, 2014*

Gene	Virus	Homology, %
HA	A/chicken/Zhejiang/S4135/2013(H7N9)	99.6
NA	A/chicken/Jilin/SD001/2014(H9N2)	99.1
PB2	A/chicken/Zhejiang/S4135/2013(H7N9)	99.9
PB1	A/chicken/Zhejiang/S4135/2013(H7N9)	99.5
PA	A/chicken/Hunan/SD015/2014(H7N9)	99.7
NP	A/Shanghai/02/2013(H7N9)	99.8
M	A/Shanghai/5190T/2013(H7N9)	99.7
NS	A/chicken/Jilin/SD001/2014(H9N2)	100

*HA, hemagglutinin; NA, neuraminidase; NP, nucleoprotein; M, matrix; NS, nonstructural; PA, polymerase basic; PB, polymerase basic.

Similar to the H7N9 viruses (*12*,*13*), A/chicken/Jilin/SD020/2014(H7N2) also has the single basic amino acid arginine in its HA cleavage site. We determined the intravenous pathogenicity index of A/chicken/Jilin/SD020/2014(H7N2) as described previously (*13*). None of the chickens showed signs of disease or died during the 10-day observation period; the intravenous pathogenicity index was 0, indicating that this H7N2 virus in chickens is nonpathogenic.

We inoculated groups of eight 6-week-old female BALB/c mice with 10^6 50% egg infectious doses of A/chicken/Jilin/SD020/2014(H7N2) and 2 H7N9 viruses, A/pigeon/Shanghai/S1069/2013, and A/Anhui/1/2013. On day 3 postinfection, 3 mice in each group were killed and their organs (nasal turbinates, lungs, spleens, kidneys, and brains) were collected for virus titration. For 14 days, the remaining 5 mice were observed for body weight changes and survival. In the mice, replication of all 3 viruses was detected in the nasal turbinates and lungs but not in other organs (Figure 1, panel A); the titers in lungs of mice infected with H7N2 virus were comparable to those in the lungs of mice infected with the human H7N9 virus A/Anhui/1/2013 and were significantly higher than those in the lungs of mice infected with the avian H7N9 virus A/pigeon/Shanghai/S1069/2013 (Figure 1, panel A). The A/Anhui/1/2013 virus–infected mice showed up to a 30% loss of body weight, and 1 mouse died during the observation period (Figure 1, panels B and C). Although none of the mice infected with the H7N2 virus or A/pigeon/Shanghai/S1069/2013 virus died during the observation period, loss of body weight was slightly more for the H7N2-infected mice than for the A/pigeon/Shanghai/S1069/2013-infected mice and the control mice (Figure 1, panel B).

We also investigated antibody responses in serum samples from chickens in the patient's village. In 55 of the 60 serum samples collected from the chickens on the patient's family farm, hemagglutination inhibition assay indicated that antibody titers against H7N2 or H7N9 virus ranged from 2 to 1,024. Only 5 of the 50 serum samples collected from the chickens on the patient's neighboring farms had hemagglutination inhibition antibody titers, which ranged from 2 to 128 (Figure 2).

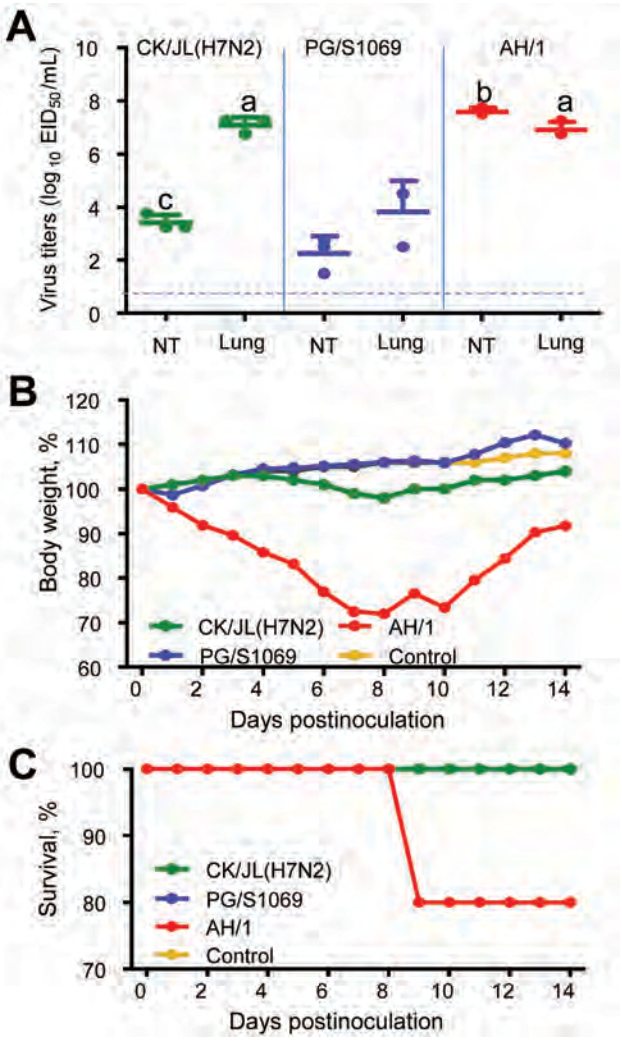

Figure 1. Replication and virulence of avian influenza A virus subtypes H7N2 and H7N9 in mice. A) Virus titers in organs of mice. The data shown are the mean ± SD for each group. Because virus was not detected from spleen, kidney, or brain of any mouse, data for these organs are not shown. a, $p<0.01$ compared with the corresponding value for the A/pigeon/Shanghai/S1069/2013-inoculated group; b, $p<0.01$ compared with the corresponding value for the A/chicken/Jilin/SD020/2014(H7N2)-inoculated and A/pigeon/Shanghai/S1069/2013-inoculated groups; c, $p<0.05$ compared with the corresponding value for the A/pigeon/Shanghai/S1069/2013-inoculated group. The dashed line indicates the lower limit of detection. NT, nasal turbinates. B) Percentages of body weight changes of mice. C) Percentages of mice that survived. CK/JL (H7N2), A/chicken/Jilin/SD020/2014(H7N2); PG/S 1069, A/pigeon/Shanghai/S1069/2013; AH/1, A/Anhui/1/2013; EID_{50}, 50% egg infectious dose.

Conclusions

The H7N2 influenza virus isolated from a chicken in Jilin Province in northern China was a novel reassortant that derived its HA, PB2, PB1, PA, NP, and M genes from the H7N9 virus that emerged in China in 2013. Although we did not find any H7N9 viruses in chickens during this investigation,

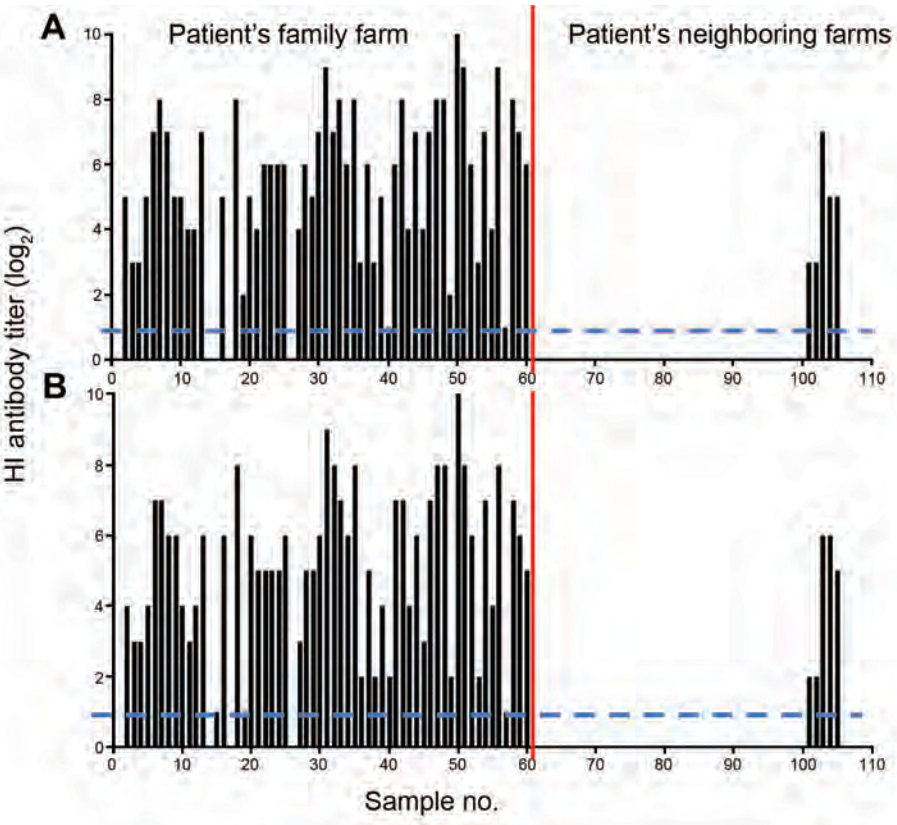

Figure 2. Antibody titers in chicken serum against H7 influenza viruses. The hemagglutination inhibition (HI) antibody titers of the serum against the H7N2 virus A/chicken/Jilin/SD020/2014(H7N2) (A) and the H7N9 virus A/pigeon/Shanghai/S1069/2013 (B) were determined with 0.5% (vol/vol) chicken erythrocytes. Each bar represents the hemagglutination inhibition antibody value of an individual serum sample. Dashed lines indicate limit of detection.

the fact that the owner of the chickens was infected with an H7N9 virus indicates that H7N9 viruses might have circulated among these chickens. The NA and NS genes of the novel H7N2 virus are closely related to those of the H9N2 virus that was isolated from chickens on the same farm, providing direct evidence that H7N9 viruses continue to evolve and reassort with H9N2 viruses in poultry in China.

H7N9 isolates from humans replicate much more efficiently and are more lethal in mice than are H7N9 isolates from birds (*13*). The mutation of glutamic acid to lysine at position 627(E627K) in PB2 contributes to this difference (*15*). The H7N2 virus does not have the PB2 627K mutation, but its replication in the lungs of mice is comparable to that of human H7N9 virus and significantly higher than that of avian H7N9 virus. These findings suggest that the continued circulation of H7 viruses in nature will enable them to acquire more mutations or new gene constellations that might increase their virulence in animals or humans.

The nonpathogenic nature of H7 viruses in poultry enables them to replicate silently in birds. The high positive ratio of antibody against H7 viruses detected by hemagglutination assay and the huge diversity of antibody levels among chickens from the H7N9 patient's farm demonstrate that the H7 viruses might have been introduced and circulated in these birds for several weeks before they were detected. These findings highlight the challenges of trying to eradicate low pathogenicity influenza subtype H7 viruses from nature and the need for continued surveillance and monitoring of H7 viruses in poultry in China.

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Differences in **Influenza** Seasonality by Latitude, Northern India

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The seasonality of influenza in the tropics complicates vaccination timing. We investigated influenza seasonality in northern India and found influenza positivity peaked in Srinagar (34.09°N) in January–March but peaked in New Delhi (28.66°N) in July–September. Srinagar should consider influenza vaccination in October–November, but New Delhi should vaccinate in May–June.

Annual and pandemic influenza are sources of considerable illness and death worldwide (*1*). Human influenza infection rates peak during the winter months in temperate regions; however, the pattern of influenza is different in tropical and subtropical areas, with year-round circulation in some areas and biannual peaks in others (*2*–*5*). The complex seasonality of influenza in the tropics complicates appropriate vaccination recommendations, particularly the timing of vaccination campaigns in tropical regions (*3*,*4*).

India has discrete seasons that vary greatly from north to south. Jammu and Kashmir, the northernmost state of India, has severe winters from December to March, whereas Delhi, the capital region, has milder winters. Sentinel surveillance for influenza in India has shown distinct influenza peaks in India (*6*–*8*). We undertook influenza surveillance during 2011–2012 in 2 cities in northern India, Srinagar and New Delhi, which are ≈500 km apart, and found evidence for discrete seasonality related to the latitudes of these cities, a finding that has implications for influenza vaccination policy and timing.

The Study

For the study, we enrolled patients who attended the outpatient clinics at the All India Institute of Medical Sciences, New Delhi, and Sheri-Kashmir Institute of Medical Sciences, Srinagar, during 2011–2012 who had influenza-like illness (ILI; defined as sudden onset or history of fever >38°C, cough or sore throat and/or rhinorrhea) (*6*). We collected 5–10 nasopharyngeal samples from these patients each week and tested them for influenza viruses by type and subtype (*9*,*10*). A confirmed case-patient was defined as a patient meeting the ILI case definition who had positive results for influenza by reverse transcription PCR. Sanger sequencing of the hemagglutinin and neuraminidase genes was completed, and consensus was used to construct a Kimura 2-parameter neighbor-joining tree (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/10/14-0431-Techapp1.pdf). Sequences from New Delhi and Srinagar were compared with those of vaccine strains and with published cognate sequences of corresponding genes (online Technical Appendix Table 1), including those from India (*10*).

Surveillance for influenza viruses revealed that overall influenza positivity was 17.6% (375 confirmed cases from 2,126 ILI patients) in Srinagar and 9.46% (239/2,526) in New Delhi (Table). Discrete winter time peaks were observed during January–March (epidemiologic weeks [EW] 1–12) in Srinagar, whereas New Delhi had peaks of influenza circulation during July 2011 and September 2012 (EW 26–36) (Figure 1). Influenza A was the predominant type in Srinagar (275/375; 72.9%), whereas influenza B dominated in New Delhi (154/239; 64.4%). Circulation of influenza A(H3N2) during the monsoon season of 2011 in New Delhi was followed by predominance of H3N2 during winter 2012 in Srinagar (Figure 1).

Phylogenetic analysis of the hemagglutinin and neuraminidase genes from selected samples from New Delhi and Srinagar revealed no notable differences between circulating viruses (online Technical Appendix Figure 1). Furthermore, circulating influenza strains were closely related to the selected influenza vaccine strains, A/California/7/2009 (H1N1), A/Perth/16/2009 (H3N2), and B/Brisbane/60/2008, which remained unchanged for 2011–2012 for the Northern and Southern Hemisphere formulations (online Technical Appendix Figure 2). The 2012–2013 Northern Hemisphere formulation changed the H3N2 strain to A/Victoria/361/2011 and the influenza B strain to B/Wisconsin/1/2010, but sequence information from 2013 circulating viruses from Srinagar was not available to assess vaccine similarity.

Analysis of meteorologic factors (i.e., rainfall, temperature, relative humidity, vapor pressure, and dew point)

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Table. Influenza surveillance, Srinagar and Delhi, India, 2011–2012

City	No. (%) persons		
	2011	2012	Total
Srinagar (34.09°N)			
Tested	768	1,371	2,139
Influenza positive	162 (21)	215 (15.7)	377 (17.6)
Influenza type*			
A(H1N1)pdm09	95 (58.6)	57 (26.5)	152 (40.3)
A(H3N2)	38 (23.5)	85 (39.5)	123 (32.6)
B	29 (17.9)	73 (34.0)	102 (27.1)
New Delhi (28.66°N)			
Tested	1,007	1,519	2,526
Influenza positive	74 (7.3)	165 (10.8)	239 (9.46)
Influenza type*			
A(H1N1)pdm09	3 (4.1)	44 (26.7)	47 (19.7)
A(H3N2)	38 (51.4)	0	38 (15.9)
B	33 (44.6)	121 (73.3)	154 (64.4)

*Percentages are of all influenza-positive test results in category.

showed that the monthly proportion of influenza positivity correlated with decreased temperature and dew point in Srinagar and with rainfall amount in New Delhi (data not shown). Cumulative data over the 2-year surveillance period revealed differences in seasonality by latitude; influenza positivity peaked during December–February in Srinagar (34.09°N) but in July–September in New Delhi (28.66°N) (Figure 2). Influenza seasonality indicates that New Delhi would likely benefit from springtime vaccination (May–June), whereas vaccination in the fall (October–November) would be better for Srinagar (Figure 2). We recently illustrated that India and most other tropical countries in Asia exhibit influenza seasonality that coincides with the monsoon season, June–October (*11*).

Conclusions

We identified discrete patterns of influenza circulation in India. In Srinagar, a city in the northernmost region of India, influenza positivity rates peaked in winter (December–March), whereas in New Delhi, a city just ≈500 km south of Srinagar, influenza peaked during the monsoon season (July–September). The winter peak in Srinagar is similar to the timing of influenza circulation observed for most countries in Europe and United States in the Northern Hemisphere (*4*,*5*). By contrast, the data on influenza seasonality in New Delhi corroborate findings which showed

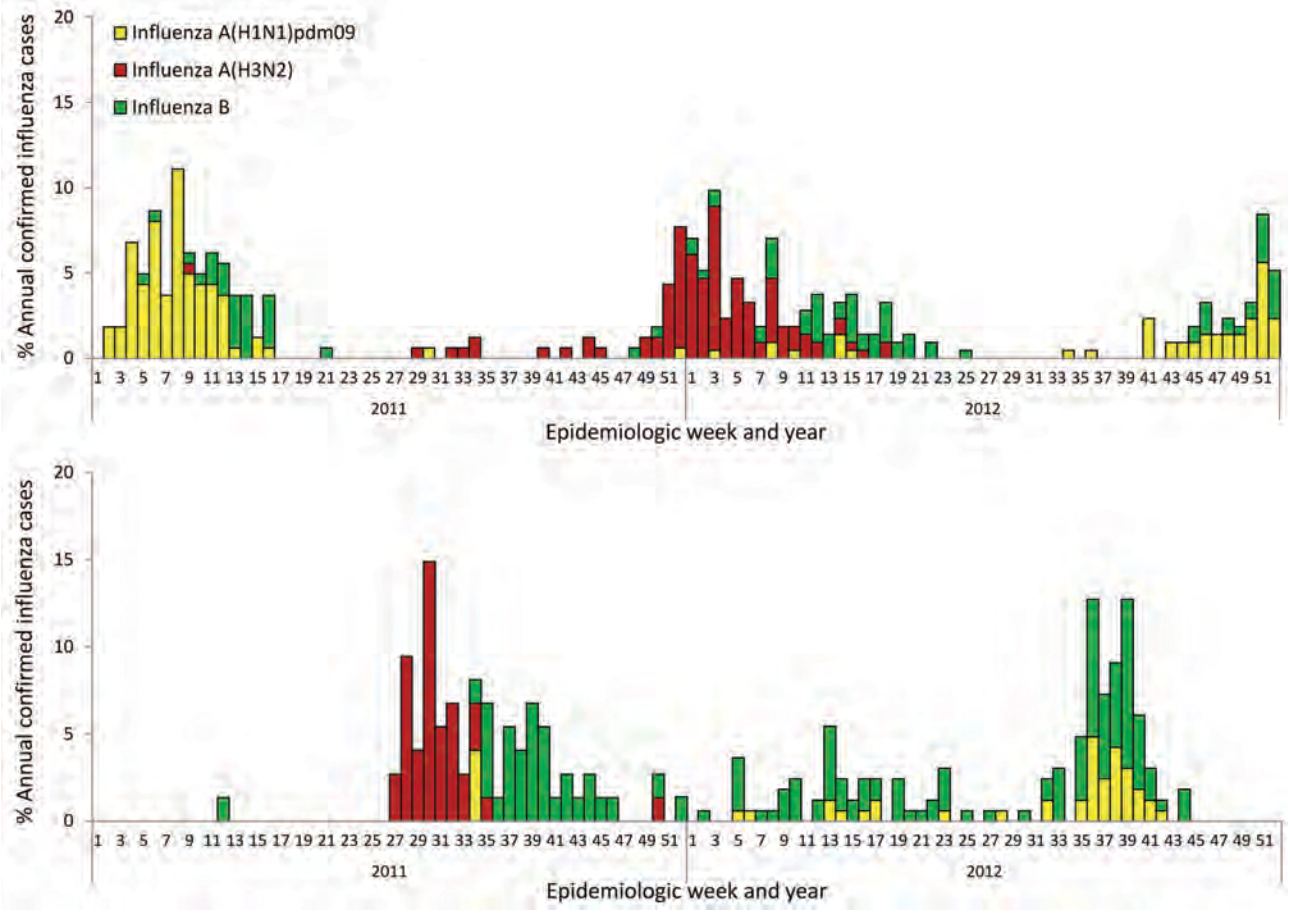

Figure 1. Weekly trends and proportion of annual numbers of positive influenza cases, by epidemiologic week and influenza type, Srinagar (A) and New Delhi (B), India, 2011–2012. Clear seasonal peaks are seen in January–March (weeks 1–16) for Srinagar and in July and September (weeks 28–40) for New Delhi.

that many countries in tropical regions (e.g., Brazil, India, Vietnam) experience high influenza transmission rates during the rainy season (*6*,*11*–*13*). Overall, influenza A and B viruses co-circulated throughout the surveillance period in Srinagar and New Delhi; however, the types and subtypes varied.

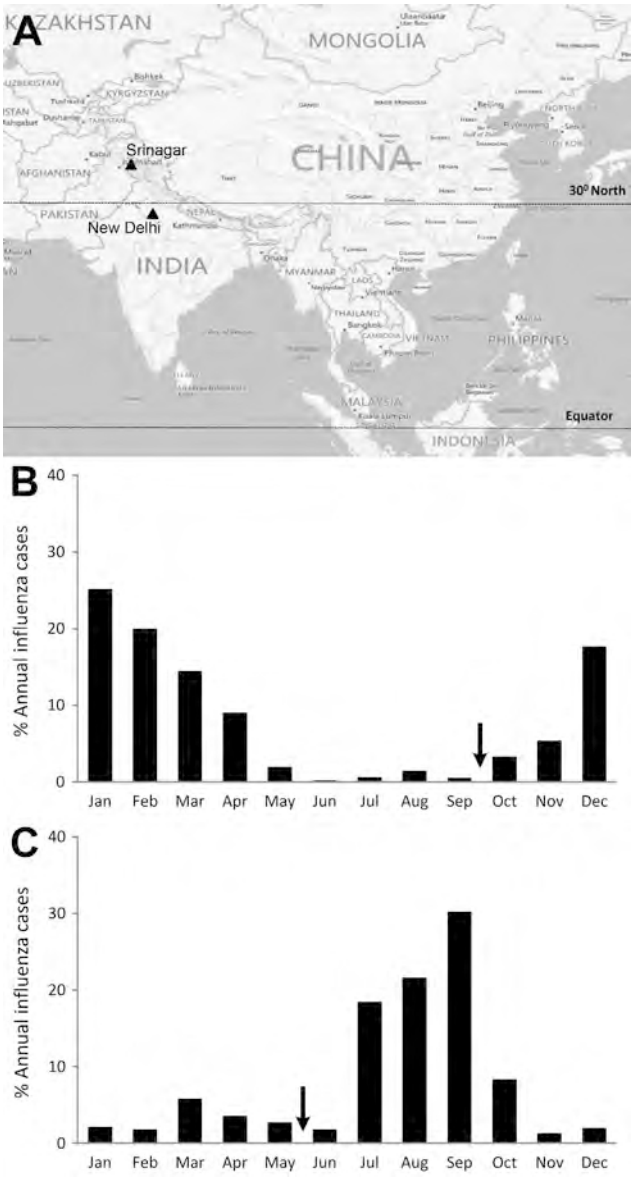

Figure 2. Comparison of latitudes of Srinagar and New Delhi, India, and distribution of influenza virus infections, 2011–2012. A) Locations of Srinagar and New Delhi (black triangles), with vertical lines indicating 30°N latitude and equator. B) Monthly distribution of cases of influenza virus infection in Srinagar (34.0°N latitude). C) Monthly distribution of cases of influenza virus infection in New Delhi (28.7°N latitude). Arrows indicate proposed vaccination timing; latitude of each city is shown. Map created in Epi-Map in Epi Info 7 (Centers for Disease Control and Prevention, Atlanta, GA, USA).

Peaks of influenza circulation in Srinagar and New Delhi show seasonal patterns that depend on factors such as temperature, rainfall, humidity, and latitude (*2*,*3*). Srinagar, at >30°N latitude, has influenza seasonality that coincides with lower temperature and low dew points during winter, whereas New Delhi, at <30°N latitude, has a peak of influenza circulation that coincides with the rainy season. Seasonal influenza activity coinciding with the humid, rainy season at lower latitudes has also been observed in large areas of Central and South America and southern Asia (*11*,*13*). In contrast, cold, dry weather was predictive of influenza peaks at higher latitudes, as observed for Srinagar. The latitude dependence of influenza circulation observed in this study is similar to such dependence observed in Brazil and China (*13*,*14*) and collectively suggests that decisions on the timing of influenza vaccination should not be based only on the hemisphere a country is in but also on the types of seasonal patterns that exist within a country (*15*). These latitudinal differences in influenza seasonality in India have implications for influenza vaccine timing and vaccine formulation.

Influenza vaccine induces a neutralizing antibody response that wanes over time. Thus, the timing of vaccination has a direct effect on vaccine effectiveness. In the northernmost part of India, peak influenza circulation occurs during the winter months; therefore, vaccination during October–November using the Northern Hemisphere vaccine formulation may be appropriate. However, this practice would not be appropriate in the Delhi metropolitan region, where influenza peaks in July–September. In addition, whereas our data points to an approximate latitude where temperate and tropical patterns for influenza peaks diverge, more robust data with multiple surveillance sites in tropical, subtropical, and temperate regions in India and China are needed to define the exact latitude points for influenza circulation patterns.

Our study has limitations. First, comparative data were available only for 2 years. Additional surveillance data for multiple years and many cities around the latitude gradient are required to further corroborate these observations. Furthermore, validation of vaccine formulation will require tracking of additional circulating influenza strains over several epidemic periods.

In summary, we identified 2 distinct seasons for influenza circulation in 2 cities in India. We recommend that policy makers in India review circulation patterns closely before implementing influenza intervention plans. Our data suggest that cities in India located north of 30°N latitude can continue vaccination in the winter, but those south of 30°N, including New Delhi, should consider vaccination in May–June (*15*). Collectively, these data should help decision makers, especially regulatory authorities, choose vaccines and vaccination schedules best suited for each region.

Acknowledgment

We acknowledge the authors and originating and submitting laboratories of the sequences from the GISAID EpiFlu Database (http://www.gisaid.org), presented in the Technical Appendix.

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Human Babesiosis, Maine, USA, 1995–2011

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We observed an increase in the ratio of pathogenic *Babesia microti* to *B. odocoilei* in adult *Ixodes scapularis* ticks in Maine. Risk for babesiosis was associated with adult tick abundance, *Borrelia burgdorferi* infection prevalence, and Lyme disease incidence. Our findings may help track risk and increase the focus on blood supply screening.

Babesiosis caused by *Babesia microti* is a potentially life-threatening parasitic infection transmitted by *Ixodes scapularis*, the deer, or black-legged, tick; it is of increasing concern as a transfusion-acquired illness (*1*,*2*). Since its recognition on Nantucket Island and Cape Cod, Massachusetts, USA, during the 1970s (*3*), human babesiosis from *B. microti* infection has become a public health threat in an increasing number of foci in the northeastern and upper midwestern United States (*1*). Risk for infection by *B. microti* remains geographically more localized than for other pathogens transmitted by *I. scapularis* ticks, such as *Borrelia burgdorferi* (*4*–*6*). This localization may be associated with dense populations of *I. scapularis* ticks and high prevalence of *B. burgdorferi* in ticks (*6*,*7*). Therefore, entomologic data may help predict risk for human babesiosis (*6*).

The presence of *B. microti*–infected ticks in Maine was first documented in 1995 from a town in which Lyme disease was endemic (*7*,*8*). The first case of human babesiosis reported in Maine occurred in 2001, 15 years after the first case of Lyme disease occurred in the state (*8*,*9*). A transfusion-associated case of babesiosis in2007 originated from a blood donor in Maine (*10*). We report on the geographic and temporal expansion of babesiosis in Maine, entomologic correlates of its emergence, and the seroprevalence of *Babesia* spp. in blood donors from the 2 southernmost coastal counties.

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The Study

We obtained the number of human cases of babesiosis and Lyme disease cases per year and county during1995–2011, from the Maine Center for Disease Control (Table 1) and obtained census data (http://quickfacts.census.gov/qfd/states/23000lk.html) for the years 2000 and 2010. We calculated incidence (cases/100,000 population) using the census for the year 2000 for 1995–2004 and the 2010 census for 2005–2011 (Table 1). Early (2001–2004) babesiosis cases occurred in the 2 southernmost coastal counties, but since 2005, cases have been reported in 8 additional counties, including 2 noncoastal counties (Figure 1). In addition to treatment with antiparasitic drugs, several severely ill patients underwent exchange transfusion. No deaths were reported.

For the period of 1995–2001, we reviewed published and unpublished data regarding presence of *Babesia* spp. in ticks tested by endpoint PCR (n = 1,433) (*7*–*9*) (Table 2). We examined data from all towns sampled, but to minimize spatial bias, we also examined data only from the town of Wells, from a site sampled in each study. Host-seeking adult ticks made up 66% of the samples, and fed nymphs made up the remainder (*8*) (Table 2). We tested for *Babesia* spp. in ticks using Feulgen stain (*7*–*9*), PCR (*7*–*9*,*11*), or both (Table 2). In Maine, *I. scapularis* ticks are known to harbor 2 *Babesia* spp: *B. odocoilei* (a deer parasite, presumed to be nonpathogenic to humans) and *B. microti* (*7*). However, 18s rRNA sequences (GenBank accession nos. AF028346, AF028343, respectively) to differentiate the species were difficult to obtain because of low DNA concentrations in some samples. Thus, for each study, we calculated the proportion of *B. microti* to *B. odocoilei* as the number of *B. microti*–positive ticks divided by the number of ticks positive for either *B. microti* or *B. odocoilei* (Table 2). Analyzing data from all towns sampled or only Wells, we observed an apparent increase in the ratio of *B. microti* to *B. odocoilei* over time that corresponded with the 2001 appearance of and subsequent increase in reported cases of babesiosis (Table 1). *B. microti* was documented in ticks only from the 2 southernmost counties.

We performed a longitudinal review of the abundance of *I. scapularis* adult ticks and prevalence of *B. burgdorferi* infection. Questing adult *I. scapularis* ticks were collected annually in the fall as previously described (*12*) at 2 long-term study sites in southern coastal Maine and episodically at other sites (Figure 2). A subset of these ticks was tested for *B. burgdorferi* infection by direct fluorescent microscopy

Table 1. Prevalence of *Borrelia burgdorferi* in *Ixodes scapularis* ticks and incidence of Lyme disease and human babesiosis, Maine, USA, 1995–2011

	Field surveys			Laboratory results				
	No. counties		No. ticks	No. ticks positive for *B.*	Lyme disease		Babesiosis	
Year	(towns)	No. ticks	collected/h	*burgdorferi*/no. tested (%)	No. cases*	Incidence	No. cases†	Incidence
1995	5 (6)	498	13	127/308 (41)	45	3.39	0	0
1996	6 (7)	595	12	131/413 (32)	63	4.74	0	0
1997	8 (8)	612	7	162/420 (39)	34	2.56	0	0
1998	3 (7)	580	16	166/399 (42)	78	5.87	0	0
1999	5 (12)	1,444	14	478/886 (54)	89	6.70	0	0
2000	6 (11)	2,390	26	599/1,164 (51)	70	5.27	0	0
2001	5 (7)	967	32	395/779 (51)	108	8.13	1	0.08
2002	3 (5)	773	42	344/669 (51)	218	16.41	2	0.16
2003	5 (9)	986	29	364/758 (48)	175	13.17	3	0.24
2004	4 (9)	799	24	326/688 (47)	224	16.86	5	0.39
2005	5 (8)	1,253	21	197/402 (49)	245	19.23	10	0.78
2006	4 (6)	974	40	342/525 (65)	338	26.53	9	0.71
2007	7 (15)	1,398	22	269/541 (50)	530	41.60	11	0.86
2008	4 (11)	610	34	192/355 (54)	909	71.34	11	0.86
2009	3 (5)	557	34	228/363 (63)	976	76.60	3	0.24
2010	5 (7)	332	14	145/251 (58)	751	58.94	5	0.39
2011	5 (7)	659	32	223/421 (53)	1,007	79.03	9	0.71

*Centers for Disease Control National Notifiable Diseases Surveillance System Lyme disease case definitions: 1995 for 1995, 1996 definition used for 1996–2001, 2011 definition used for 2002–2011.
†CDC National Notifiable Diseases Surveillance System 2011 babesiosis case definition.

(*12*). Table 1 shows upward trends in abundance of adult *I. scapularis* ticks, prevalence of *B. burgdorferi* infection, and incidence of Lyme disease and babesiosis.

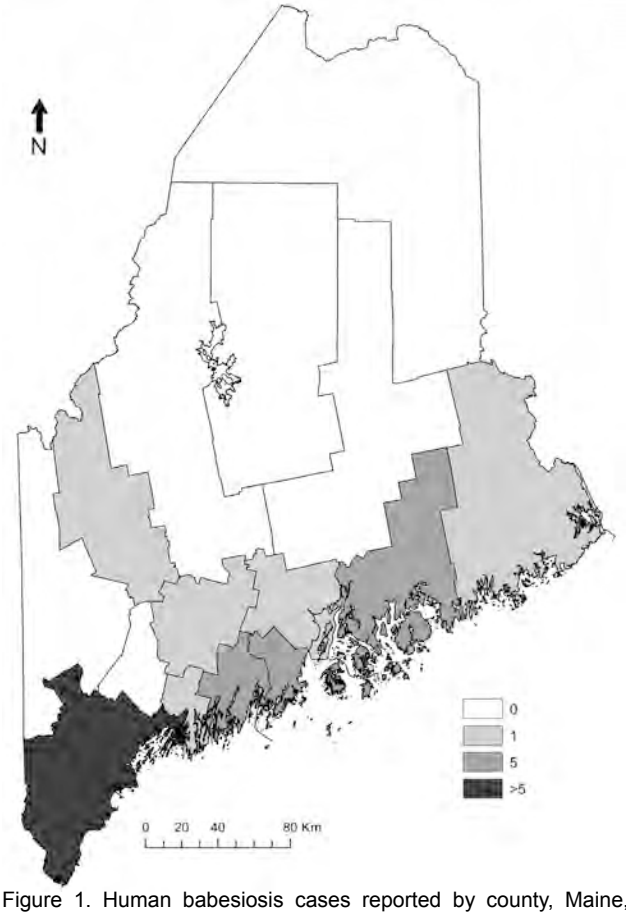

Figure 1. Human babesiosis cases reported by county, Maine, USA, 2001–2011.

Mather et al. (*5*) used logistic regression to demonstrate that abundance of questing nymphal *I. scapularis* ticks (nymphs per hour) of 19–135/hour in wooded areas predicted a ≥20% probability of human babesiosis cases in Rhode Island. Using SAS 9.2 (SAS Institute Inc., Cary, NC, USA), we examined the 20% probability of a babesiosis case as a function of adult ticks collected per hour, *B. burgdorferi* infection prevalence in adult ticks, or Lyme disease incidence.

We categorized babesiosis cases as present or absent and calculated the number of adult ticks collected per hour, *B. burgdorferi* infection prevalence, and Lyme disease incidence by county and year. We evaluated each univariate model by using the Wald χ^2_W and assuming models to be significant at χ^2_W $p \leq 0.05$; and the Hosmer and Lemeshow goodness-of-fit test χ^2_{HL}, assuming suitable fit at χ^2_{HL} $p \geq 0.10$. For the models, all $\chi^2_W \geq 8.4$ and $p \leq 0.004$, and all $\chi^2_{HL} \leq 11.3$ and $p \geq 0.13$. A 20% probability of ≥1 babesiosis case was predicted during years in which abundance of adult ticks collected exceeded 17 per hour (n = 83; 95% CI 12.1%–31.7% for a 20% probability), when *B. burgdorferi* infection prevalence among adult ticks exceeded 34% (n = 74; 95% CI 11.6%–33.6%), or when Lyme disease incidence exceeded 58 cases/100,000 population (n = 272; 95% CI 13.6%–27.9%).

With approval from the Maine Medical Center Institutional Review Board, and in collaboration with Coral Blood Services, Inc., (Scarborough, ME, USA) blood samples from healthy donors were de-identified and screened for *Babesia* antibodies during July–December 2010. Using postal codes, we selected 311 donors from the 2 southernmost Maine counties (Cumberland and York) where *I. scapularis* ticks are more abundant than elsewhere in the state

Table 2. Emergence of *Babesia microti* in *Ixodes scapularis* ticks, Maine, USA, 1995–2011

			All towns sampled, N = 90*		Town of Wells	
			PCR	Sequenced	PCR	Sequenced
Year(s) (ref.)	Sample type	PCR primers†	No. ticks positive for *Babesia* spp./no. tested	No. *B. microti*/no. sequenced (%)	No. ticks positive for *Babesia* spp./no. tested	No.*B. microti*/no. sequenced (%)
1995–96 (*7*)	Questing adult tick (salivary glands)	PIRO-A/B	28/83	1/25 (4)	11/30	1/10 (10)
1995–1997 (*8*)	Partially engorged nymphal and adult ticks on rodent, dog, cat, and human hosts (salivary glands)	PIRO-A/B	65/455	3/65 (5)	18/148	2/21 (9)
1995–1998 (this study)	Questing adult ticks (salivary glands)	PIRO-A/B	24/208	0/24	8/49	0/8
2003 (*9*)	Questing adult ticks (tick bodies)	PIRO-A/B	15/100	7/15 (47)	15/100	7/15 (47)
2006–07, 2010–11 (this study)	Questing adult ticks (tick bodies)	Bab-1/4 (2006–07), PIRO-A/B	55/728	7/8 (88)	18/126	6/6 (100)

*During 1995–2011, *B. microti* was found only in the southern coastal towns of Kittery, Wells, and Cape Elizabeth; *B. odocoilei* was found in Cape Elizabeth, Wells, and 29 additional, mostly coastal, towns.
†The primer pair PIRO-A, PIRO-B targets the 18S rRNA gene, 408 bp for *B. odocoilei*/ 437 bp for *B. microti* (7); the primer pair Bab-1, Bab-4 targets the 18S RNAgene, 238 bp for *Babesia* spp. (*11*).

(*13*). Samples were tested via indirect immunofluorescent antibody (IFA) tests as per Krause et al. (*14*) by using serum diluted 1:64. Of 311 blood samples, 10 (3.2%) tested positive for *Babesia* antibody (IgG), which is in the middle of the range for blood donors in the northeastern United States (0.2% in areas where babesiosis is not endemic to 7.3% in highly disease-endemic areas [*15*]). When serum samples were IFA-positive, DNA was extracted from the corresponding whole blood sample at the Yale University School of Public Health laboratory for real-time PCR (Peter Krause, proprietary protocol). *B. microti* DNA was not detected in any of the 10 antibody-positive serum specimens.

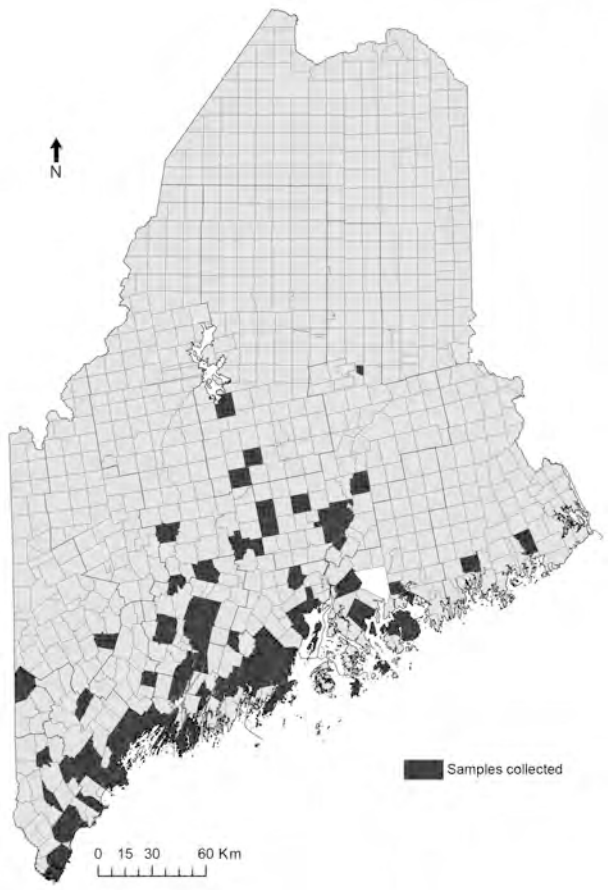

Figure 2. Distribution of towns sampled for questing adult *Ixodes scapularis* ticks, Maine, USA, 1995–2011.

Conclusions

Early studies revealed a higher ratio of presumed nonpathogenic *B. odocolei* to *B. microti* in areas where these species co-exist. The observed temporal shift to *B. microti* in questing adult and fed nymphal *I. scapularis* ticks in this review could be related to ecologic change, or to sampling bias (*7*). Adult *I. scapularis* tick abundance, *B. burgdorferi* infection prevalence among these ticks, and Lyme disease incidence may assist in the prediction of human babesiosis risk. The association observed between number of babesiosis cases and adult *I. scapularis* tick abundance was on a scale similar to that found by Mather et al. (*5*), although that study used nymphal tick abundance. IFA positivity for *Babesia* spp. IgG antibody at >1:64 has a high specificity (90%–100%) (*14*), but its predictive value is uncertain because disease prevalence is unknown for healthy blood donors. As human babesiosis emerges in Maine, refined models correlating babesiosis cases with tick abundance or Lyme incidence, or both, may help track geographic risk and permit targeted screening of the blood supply.

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Prevalence of *Arcobacter* Species among Humans, Belgium, 2008–2013

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We examined fecal samples from 6,774 patients with enteritis in Belgium, 2008–2013. Members of the genus *Arcobacter* were the fourth most common pathogen group isolated, and the isolation rate was higher than previously reported. Culturing *Arcobacter* in a microbiology laboratory is feasible and should thus be tested for in cases of diarrheal disease.

Campylobacteriosis is the most frequently reported zoonosis in industrialized countries with an increasing incidence during 2007–2011 (*1*). In this study, bacteria of the *Arcobacter* genus, which is closely related to the *Campylobacter* genus, comprised the fourth most common pathogenic group isolated from stool specimens of patients with acute enteritis in Ghent, Belgium.

Bacteria species of the genus *Arcobacter* were first isolated from aborted bovine and porcine fetuses in 1977 (*2*). Based on similar phenotypic characteristics, they were originally classified as aerotolerant *Campylobacter* spp., until a separate genus was introduced in 1991 (*2*). Since then, 18 species have been identified and new species are pending. Members of the genus *Arcobacter* are aerotolerant gram-negative bacteria and able to grow at temperatures <30°C, which differentiate them from the *Campylobacter* species. The species *A. butzleri*, *A. cryaerophilus*, *A. skirrowii*, *A. cibarius*, *A. thereius*, and *A. trophiarum* have been identified in livestock worldwide and have been isolated from food of animal origin. Though these species have been associated with illness in farm animals, they are also known to colonize healthy animals (*3*).

Arcobacter have been classified as emergent pathogens by the International Commission on Microbial Specifications for Foods (*4*). Currently, 3 species have been reported to infect humans. Contaminated drinking water and raw or undercooked food that is eaten or handled are the most frequent sources of human infection (*5*,*6*). An association of *A. butzleri* and *A. cryaerophilus* with enteritis, colitis, and septicemia has been proposed in epidemiologic studies, outbreak reports, and case reports (*7*), although *A. cryaerophilus* can also be present in healthy humans (*8*). More recently, *A. skirrowii* has been implicated as the causal agent of bacteremia and colitis in individual case reports (*9*).

Information about transmission, colonization in the gut, and virulence in humans of members of the *Arcobacter* genus is insufficient. The presence of virulence genes and their distribution within the genus have been proven, but their role in pathogenicity in selected host groups has not yet been demonstrated (*10*).

Most laboratories do not use appropriate culture methods or conditions to detect species other than *Campylobacter jejuni* and *C. coli* from feces. Furthermore, identification of *Campylobacter* and related organisms to species level is not always performed or is executed with phenotypic methods, leading to conflicting results. Hence, data on the incidence and clinical importance of *Arcobacter* remain scarce. The aims of this study were to assess the feasibility of *Arcobacter* culture in a clinical microbiology laboratory and to determine the recent prevalence of *Arcobacter* spp. in humans with gastrointestinal disease in Belgium.

The Study

Fecal samples were collected from outpatients and patients with symptoms of enteritis who were admitted for <72 hours to the Sint-Lucas Hospital in Ghent, Belgium, during 2008–2013. All age groups were represented. We examined the fecal samples macroscopically for consistency and presence of blood and mucus and microscopically for leukocytes and cultured them for all common bacterial pathogens. Feces of patients <2 years of age were also tested for rotavirus and enteric adenovirus, and those of adult patients with loose or mucous-containing stools were also tested for toxigenic *Clostridium difficile*. Samples were tested for parasites on clinical demand only.

We cultured for *Arcobacter* using an isolation method for fecal samples carried out in veterinary medicine that was previously validated for use on human feces (*11*): 1 g of feces was inoculated into a liquid selective broth (24 g/L *Arcobacter* broth with 50 mL lysed, defibrinated horse blood and an antimicrobial supplement of 16 mg/L cefoperazone, 10 mg/L amphotericin B, 100 mg/L 5-fluorouracil, 64 mg/L trimethoprim, and 32 mg/L novobiocine [Oxoid, Cambridge, United Kingdom]) and incubated for 24 h at 25°C in a microaerobic atmosphere of 6% O_2, 7% CO_2, 7% H_2, and 80% N_2 (Advanced Instruments,

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Inc., Norwood, MA, USA). Then, 40 μL of enriched broth was plated onto a solid *Arcobacter*-selective medium with the same composition as the broth but with the addition of 12 g/L agar technical no. 3 (Oxoid) and without the horse blood. The plates were incubated for 72 h at 25°C in a microaerobic atmosphere and examined daily. *Arcobacter* colonies were detected by screening the selective transparent agar as described with Henry transillumination microscopy for bluish colonies (*11*). Definitive identification was performed by a genus- and species-specific multiplex PCR, and subsequently, by amplification fragment length polymorphism (*12*,*13*).

Of the 8,994 eligible samples received during the study period, 6,774 (75.31%) samples were cultured for *Arcobacter*; 2,220 samples (24.68%) were excluded because of insufficient sample size. Of samples from the study population, *Campylobacter* spp. were isolated in 380 (5.61%), *Salmonella* spp. in 138 (2.04%), toxigenic *C. difficile* in 109 (1.61%), *Aeromonas* spp. in 16 (0.24%), and *Yersinia enterocolitica* in 13 (0.19%). Other gastrointestinal pathogens isolated in lower numbers included *Shigella* spp. (9 samples, 0.13%) and *Plesiomonas* spp. (3 samples, 0.05%).

Arcobacter spp. were isolated in samples from 89 patients (1.31%), ranking members of this genus as the fourth most commonly isolated pathogen group in the study. This ranking is comparable to those of other reports, although a higher isolation rate was obtained during this study (*14*,*15*). The distribution frequency of pathogens and of *A. butzleri* versus *A. cryaerophilus*, 49 (0.72%) and 38 (0.56%) isolates, respectively, is shown in Table 1. *A. skirrowii* was not recovered, but the first 2 known isolates of *A. thereius* (0.03%) from humans were documented. In the excluded population (n = 2,220), comparable recovery rates of *Campylobacter* spp. (6.53%), *Salmonella* spp. (2.88%) and *C. difficile* (2.07%) were observed. In 6 patients whose samples were positive for *Arcobacter*, a second pathogen was isolated: 1 patient was positive for *Salmonella* spp., samples from 2 patients showed *Campylobacter* spp. and *A. butzleri*, and toxigenic *C. difficile* was detected in 2 patients who tested positive for *A. butzleri* and in 1 patient who tested positive for *A. cryaerophilus*.

We collected retrospective data about age, hospital stay, presence of diarrhea, clinical diagnosis of enteritis, colonoscopy results, and underlying disease for the positive-culture patient group. We assessed differences between study populations and results using *t*-test and X^2 statistical methods as appropriate.

The mean age of the included population (29 years) differed significantly ($p<0.01$) from the excluded group (22 years), because of an excess of infants from whom sample material was insufficient in the latter group. The positive-culture patient group had a mean age of 42 years and demonstrated a significantly older age distribution pattern ($p<0.01$) (Figure). The consistency of fecal samples was not predictive ($p = 0.62$) for the presence of *Arcobacter*. Within the *Arcobacter*-positive group, the mean age of patients whose specimens shed *A. butzleri* (49 years) was significantly higher (15 years, $p<0.01$) than that of patients whose specimens shed *A. cryaerophilus* (34 years). Presence of *A. butzleri* in patients admitted to the hospital and in outpatients was not significantly linked with underlying disease ($p = 0.12$). *A. cryaerophilus* was more frequently observed in outpatients who had uncomplicated gastroenteritis than in hospitalized patients with coexisting conditions ($p<0.001$) (Table 2).

Table 1. Distribution of study population and number (%) of bacterial gastrointestinal pathogens during the study periods 2008–2010 and 2012–2013, Belgium*

Characteristics	2008 (%)	2009 (%)	2010 (%)	2012 (%)	2013 (%)	5-y period (%)
Eligible samples	1,819	1,843	1,612	2,229	1,491	8,994
Included	1,375 (76)	1,374 (75)	1,112 (69)	1,768 (79)	1,145 (77)	6,774 (75)
Excluded	444 (24)	469 (25)	500 (31)	461 (21)	346 (23)	2,220 (25)
Pathogens identified						
Included patients						
Campylobacter spp.	64 (4.7)	54 (3.9)	68 (6.1)	85 (4.8)	109 (9.5)	380 (5.6)
Salmonella spp.	29 (2.1)	32 (2.3)	28 (2.5)	26 (1.5)	23 (2.0)	138 (2.0)
Clostridium difficile†	26 (1.9)	19 (1.4)	17 (1.5)	18 (1.0)	29 (2.5)	109 (1.6)
Arcobacter spp.	18 (1.3)	12 (0.9)	18 (1.6)	17 (0.9)	24 (2.1)	89 (1.3)
Arcobacter butzleri	6 (0.4)	7 (0.5)	11 (0.9)	7 (0.4)	18 (1.6)	49 (0.7)
Arcobacter cryaerophilus	12 (0.9)	5 (0.4)	5 (0.4)	10 (0.7)	6 (0.5)	38 (0.6)
Arcobacter thereius	0	0	2 (0.1)	0	0	2 (0.03)
Aeromonas spp.	2 (0.1)	3 (0.2)	6 (0.5)	1 (0.1)	4 (0.3)	16 (0.2)
Yersinia enterocolitica	1 (0.1)	4 (0.3)	1 (0.1)	2 (0.1)	5 (0.4)	13 (0.2)
Shigella spp.	2 (0.1)	2 (0.1)	3 (0.3)	2 (0.1)	0	9 (0.1)
Plesiomonas spp	0	2 (0.1)	0	0	1 (0.1)	3 (0.04)
Excluded patients						
Campylobacter spp.	20 (4.5)	23 (4.9)	39 (7.8)	27 (5.9)	36 (10.4)	145 (6.5)
Salmonella spp.	20 (4.5)	14 (3.0)	15 (3.0)	11 (2.4)	4 (1.2)	64 (2.9)
Clostridium difficile†	8 (1.8)	16 (3.4)	7 (1.4)	8 (1.7)	7 (2.0)	46 (2.1)

*The study was interrupted for 1 y in 2011.
†Toxigenic strains only.

Table 2. Microbiological and clinical details for 86 patients whose fecal samples contained *Arcobacter* spp., Belgium, 2008–2013

	Arcobacter spp. samples, N = 89		
	A. butzleri, n = 49* (55%)	*A. cryaerophilus*, n = 38 (43%)	*A. thereius*, n = 2 (2%)
Characteristic	No. (%)	No. (%)	No. (%)
Fecal consistency			
Solid	18 (37)	16 (42)	0
Semisolid-liquid	25 (51)	22 (58)	1 (1)
Mucous	5 (10)	0	1 (1)
Bloody	1 (2)	0	0
Clinical status			
Ambulatory	19 (39)	32 (84)	1 (1)
Hospitalized	30 (61)	6 (16)	1 (1)
Clinical syndromes			
Acute gastroenteritis	19 (39)	30 (79)	1 (1)
Coexisting medical condition	30 (61)	8 (21)	1 (1)
Chronic colitis	8 (15)	2 (5)	1 (1)

*49 samples from 46 patients.

Conclusions

Arcobacter species were the fourth most common pathogen group isolated from fecal samples from persons with acute enteric disease. The high isolation rate could possibly be explained by the inclusion of an outpatient population, local indications for sampling, and the use of an enrichment culture method. The *Arcobacter*-positive patients tended to belong to older age groups. No notable association of *A. butzleri* enteritis with coexisting conditions was observed. The feasibility of selective culturing *Arcobacter* from fecal material in a routine microbiology hospital laboratory was confirmed, but the slow turnaround times for culture results show a need for optimization of methods. *Arcobacter* species should be considered and tested for in cases of diarrheal disease.

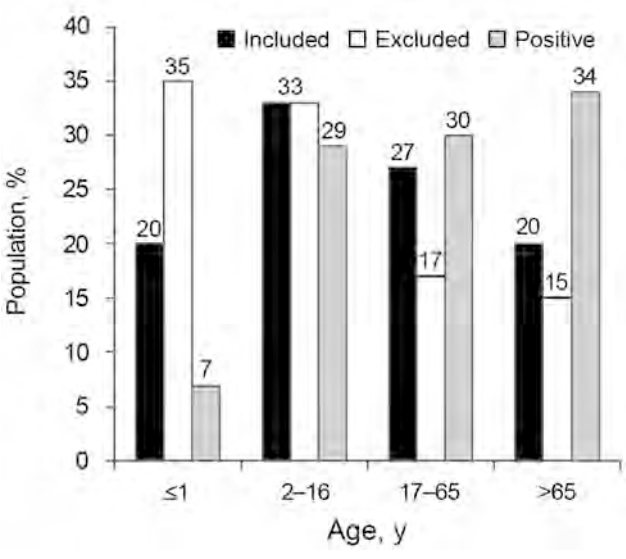

Figure. Age distribution of study population for detection of *Arcobacter* spp. in patients with acute enteritis, 2008–2013, Belgium. Black bars indicate percentage of age group included, white bars indicate percentage of patients excluded from the study, and gray bars indicate percentage of patients whose samples tested positive for *Arcobacter* spp.

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Evidence of Recombinant Strains of Porcine Epidemic Diarrhea Virus, United States, 2013

Peng-Fei Tian, Yu-Lan Jin, Gang Xing, Ling-Ling Qv, Yao-Wei Huang,[1] and Ji-Yong Zhou[1]

To investigate the evolutionary process by which porcine epidemic diarrhea virus (PEDV) in the United States hypothetically descended from strains in China, we analyzed PEDV-positive samples collected in China during January 2012–July 2013. Recombination in 2 strain sublineages was likely associated with identification of PEDV in the United States in 2013.

Porcine epidemic diarrhea virus (PEDV) is an alphacoronavirus that causes enteric disease in swine. The disease, PED, is characterized by acute vomiting and watery diarrhea and causes high mortality rates in newborn piglets (*1*). PED was first reported in 1971 in the United Kingdom and was soon identified in many European and Asian countries (*1*,*2*). Variant PEDV strains that were fatal to young pigs, initially isolated during late 2010 in China and Southeast Asia (*3*,*4*) and in May 2013 in the United States (*5*,*6*), have posed a serious threat to the pork industry. Incidence of PEDV-associated large–scale outbreaks of diarrheal disease in China was reported at 80%–100% in suckling piglets (*3*,*7*) and outbreaks in the United States had spread to 25 states by February 2014 (http://www.aasv.org/news/story.php?id=7038), causing numerous deaths in neonatal piglets (*5*,*6*,*8*,*9*).

How the virus entered the United States remains unknown. A phylogenetic analysis based on available full-length genomic PEDV sequences indicated that all PEDV strains were classified into 2 distinct genogroups: G1 and G2 (*6*). PEDV field strains isolated before 2010 and the derived vaccine strains were in the G1 genogroup, whereas all the new PEDV strains isolated since 2011 in China and the United States (US PEDV) are in G2. The US PEDV sequences were >99% identical to strains found in China in the subgroup G2a, suggesting their origin. In particular, the US PEDV are most closely related to strain AH2012, which was isolated in eastern China and was proposed to have come from multiple recombination events among G2 lineages of PEDV (*6*). Divergence of PEDV is driven by genetic recombination, as in other coronaviruses (*10*). Details of recombination events in the process are needed to investigate origins. To investigate the evolutionary process by which US PEDV strains hypothetically descended from precursors in China, we conducted a molecular epidemiologic analysis using PEDV-positive samples collected from eastern China since 2012.

The Study

A total of 169 fecal and intestinal samples were collected from pigs with typical PED symptoms on 26 farms in 4 provinces of eastern China during January 2012–July 2013. The rate of PEDV-positive samples was 56.8% (96/169) as had been determined by using reverse transcription PCR (RT-PCR) specific for the spike (S) gene (*11*). From the positive samples, we selected 24 representative samples (Table) to examine. Using RT-PCR, we determined the sequences of the full-length genomic cDNA for the strain CH/ZJCX-1/2012, identified the spike (S) gene for strains CH/ZJQZ-2w/2012 and CH/ZJDX-1/2012, and identified the region encoding structural protein genes by an order of 5′-S-ORF3-E-M-N-3′ (5′-spike protein–open reading frame 3–envelope–membrane–nucleoprotein-3′) for the remaining 21 strains. All primers were designed based on the PEDV MN strain (GenBank accession no. KF468752). We purified and cloned PCR products into a vector using TA cloning. We used Vector NTI software (http://www.lifetechnologies.com/us/en/home/life-science/cloning/vector-nti-software.html) to assemble and analyze the sequences. We performed multiple alignments of S-ORF3-E-M-N, S, ORF3, M, N, and full-length genomes with available sequences from Asia and the United States (*5*,*6*,*8*,*12*) and performed phylogenetic analyses using the MEGA5.2 program (http://www.megasoftware.net/) with the neighbor-joining method.

Similar to most of the sequences recently documented in PEDV strains in China and the United States, the S genes of the 24 samples have a 4,161-nt sequence that, compared with the prototype CV777 strain, shows 97.9%–100% sequence identities, and contain 2 notable insertions at amino acids (aa) 56–59 (IGEN) and 139 (N) and a deletion of 2 aa (GK) at aa positions 160 and 161 at the N terminus (*6*). A phylogenetic analysis comparing S genes showed

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[1]These authors were co-principal investigators.

Table. Summary of 24 representative porcine epidemic diarrheal virus sequences in China determined in this study, 2012–2013

Strain/year	Province of collection	Sequencing region*	GenBank accession no.
CH/ZJCX-1/2012	Zhejiang	Full-length	KF840537
CH/ZJHY-2/2012	Zhejiang	S-ORF3-E-M-N	KF840538
CH/ZJJS-Z/2012	Zhejiang	S-ORF3-E-M-N	KF840539
CH/ZJJS-1Z/2012	Zhejiang	S-ORF3-E-M-N	KF840540
CH/JXZS-3H/2012	Jiangxi	S-ORF3-E-M-N	KF840541
CH/JXZS-2H/2012	Jiangxi	S-ORF3-E-M-N	KF840542
CH/ZJXS212/2012	Zhejiang	S-ORF3-E-M-N	KF840543
CH/ZJHZHY-6/2013	Zhejiang	S-ORF3-E-M-N	KF840544
CH/JXJDZ-F/2012	Jiangxi	S-ORF3-E-M-N	KF840545
CH/ZJJS-2Z/2012	Zhejiang	S-ORF3-E-M-N	KF840546
CH/JXZS-3L/2012	Jiangxi	S-ORF3-E-M-N	KF840547
CH/JXZS-1223L/2012	Jiangxi	S-ORF3-E-M-N	KF840548
CH/SDZD-1/2012	Shandong	S-ORF3-E-M-N	KF840549
CH/SDZD-2/2012	Shandong	S-ORF3-E-M-N	KF840850
CH/HuBWHYQ/2012	Hubei	S-ORF3-E-M-N	KF840851
CH/ZJQZ-2/2012	Zhejiang	S-ORF3-E-M-N	KF840852
CH/ZJHZ-1C/2012	Zhejiang	S-ORF3-E-M-N	KF840853
CH/ZJHZ-2C/2012	Zhejiang	S-ORF3-E-M-N	KF840854
CH/JXJDZ-1/2012	Jiangxi	S-ORF3-E-M-N	KF840855
CH/ZJJS-4X/2012	Zhejiang	S-ORF3-E-M-N	KF840856
CH/ZJQZ-2w/2012	Zhejiang	S	KF840857
CH/ZJDX-1/2012	Zhejiang	S	KF840858
CH/JSZL-2/2013	Jiangxi	S-ORF3-E-M-N	KF840861
CH/JSZL-3/2013	Jiangxi	S-ORF3-E-M-N	KF840862

*S, spike protein; ORF, open reading frame; E, envelope; M, membrane; N, nucleoprotein.

that, based on the complete genome (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/20/10/14-0338-Techapp1.pdf), all 24 strains were classified into the same group corresponding to G2. However, it is notable that the Chinese sublineage (branch) most closely related to the US PEDV strains did not include the AH2012 strain. Instead, this sublineage contained the strain CH/ZMDZY/11 and 4 other strains determined in this study. Analyses of the phylogenetic trees constructed on the basis of the S-ORF3-E-M-N genes (Figure), ORF3 or M (online Technical Appendix Figure 1) also indicated that the AH2012 strain was not closely related to the US branch, relative to the sublineage represented by strains CH/ZMDZY/11, CH/HuBWHYQ/2012, CH/JXZS-1223L/2012, and CH/JXZS-3L/2012 (designated the ZMDZY sublineage hereafter; Figure). The exception is the N gene–based tree, in which the AH2012 was grouped more closely to the sublineage associated with the United States than the strains in the designated ZMDZY sublineage (online Technical Appendix Figure 1).

The relationship of the AH2012 strain with the 33 PEDV strains identified in China, the United States, South Korea, and Belgium (online Technical Appendix Figure 1) in nonstructural protein genes was also determined by generation of 3 phylogenetic trees based on ORF1ab, ORF1a, and ORF1b genes, respectively. In accordance with the results from the N gene and the PEDV genotyping based on the full-length genomes (*6*), the AH2012 strain in these trees was most closely related to the US strains (online Technical Appendix Figure 1). Therefore, the strains AH2012 and CH/ZMDZY/11 displayed different phylogenetic relationships in different genome regions. Overall, the AH2012 strain was clustered closely with the US strains in the ORF1ab and the N gene region, whereas the ZMDZY sublineage was clustered closely with the US strains in the S-ORF3-E-M region.

To accurately determine how the US strains are related to strains AH2012 and the ZMDZY sublineage, we performed a recombination analysis using the Recombination Detection Program and available PEDV sequences (*13*). We used a multiple-comparison–corrected p-value cutoff of 0.05 in all methods. Recombination events were identified by 6 methods (Recombination Detection Program, GENECONV, BOOTSCAN, MaxChi, CHIMAERA, and SISCAN) when the US PEDV sublineage represented by the MN strain was used as a query. By bootstrap analysis, 3 putatively major recombination breakpoints were detected at nucleotides 6699, 21840, and 26882 (online Technical Appendix Figure 2), which generated 2 regions: 1 covered the 3′ half of ORF1a, complete ORF1b, and the N terminus of the S (first 1,207 nt); the other spanned partial S, ORF3, E, M, and partial N (first 504 nt) between the strain AH2012 (as the major parent) and the ZMDZY sublineage (as the minor parent). Although the second region (partial S-ORF3-E-M-partial N) of the US strains is associated with the ZMDZY sublineage, the source of genetic material in this region is not known, because none of the PEDV strains in this sublineage had a highly identical sequence to the consensus sequence of the US strains. It is possible that the other recombination breakpoints exist within the S gene, according to the bootstrap supports in this region, which may be determined by future study with available

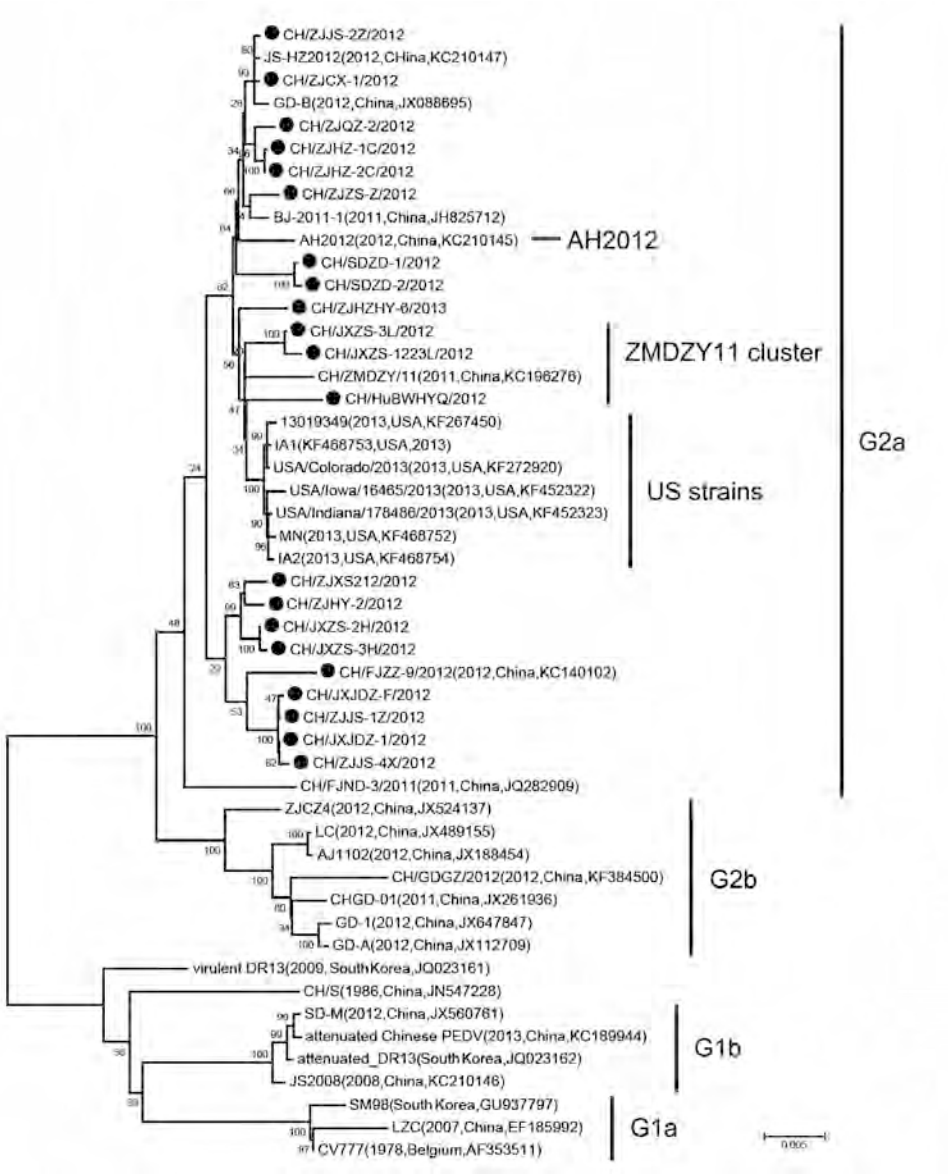

Figure. Phylogenetic analysis of newly determined and available porcine epidemic diarrhea virus strains based upon the S-ORF3-E-M-N (5′-spike protein–open reading frame 3–envelope–membrane–nucleoprotein-3′) nucleotide sequences. The tree was constructed by the neighbor-joining method. Bootstrap values are indicated for each node from 1,000 resamplings. The names of the strains, years and places of isolation, GenBank accession numbers, and genogroups proposed by Huang et al. (*6*) are shown. Black solid circles indicate the 21 PEDV strains in this genetic series. Scale bar represents nucleotide substitutions per site.

new sequence data. We showed that the emergent US PEDV strains are possibly descendent of 2 major lineages derived from the ZMDZY sublineage and AH2012 through recombination.

Conclusion

Our study provides further information on the origin of the US PEDV in 2013. We identified 21 S-ORF3-E-M-N genes, 2 S genes, and 1 full-length genomic cDNA of PEDV from PEDV-positive samples collected in eastern China. Comparative genomic, phylogenetic, and recombination analyses using new and known sequence data demonstrated that the AH2012 strain is likely not the direct progenitor of emergent US PEDV strains during 2013. It is possible that replacement of a region within the partial S-ORF3-E-M-partial N region of the AH2012 strain with a corresponding fragment close to the ZMDZY sublineage (including several newly identified strains) resulted in a recombinant strain related to emergence of this virus in swine in the United States. Other unidentified recombination events and accumulation of adapted mutations within the structural protein genes were also likely involved in this process.

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Autochthonous Melioidosis in Humans, Madagascar, 2012 and 2013

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Melioidosis is an often fatal infectious disease affecting humans and animals in the tropics. Only sporadic cases have been reported from Africa and the Indian Ocean region. We describe 2 confirmed autochthonous cases of human melioidosis in Madagascar, both from novel genotypes of *Burkholderia pseudomallei*.

Melioidosis is an often fatal infectious disease caused by the soil bacterium *Burkholderia pseudomallei*. Incidence rates are increasing in regions of Southeast Asia and northern Australia to which it is endemic, and cases are increasing from the tropics worldwide (*1*). Little is known about the epidemiology of melioidosis in the Indian Ocean region and Africa, where only sporadic cases are reported (*2*–*5*). We report 2 melioidosis cases in residents of the city of Mahajanga, the capital of the Mahajanga Province of Madagascar (Figure). These cases were identified within an ongoing project to document melioidosis in Madagascar conducted by the Pasteur Institute in Antananarivo.

Mahajanga, a favorite tourist destination in Madagascar, is located on the country's northwestern coast on the Mozambique Channel (15°43′S, 46°18′E), 550 km from Antananarivo, the capital of Madagascar. Its population is 200,000 persons.

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Case Reports

Case-patient 1 was a 52-year-old male rural rice farmer, who had diabetes. He was admitted to Androva University Hospital in Mahajanga in July 2012 with systemic sepsis, from which he died 3 days later despite treatment with ceftriaxone. Chest radiographs showed opacity in the lower left lung and pleural effusion. Abdominal ultrasound showed hepatomegaly and splenomegaly with small hypoechogenic lesions in the spleen consistent with abscesses. Culture media used to recover organisms from the blood were Ashdownagar supplemented with gentamicin 4 mg/L (*6*), chocolate agar, and blood agar incubated for 24 h at 37°C after inoculation. A blood culture (Hemoline Performance Diphasique, bioMérieux, Marcy l'Etoile, France) taken the day after admission was positive for *B. pseudomallei*; *B. pseudomallei* was suspected 8 days later on the basis of a biochemical phenotype (82% identity) using API20NE, 1154576 (bioMérieux).

Case-patient 2 was a 45-year-old male rice, sugar cane, and tobacco farmer, who had diabetes. He was admitted to Androva University Hospital in May 2013 with a recurrent fever and a history of furunculosis for several months. A week after admission, his condition deteriorated; he had progressive sepsis and hepatic failure despite therapy with ceftriaxone, ciprofloxacin, metronidazole, and gentamicin. Ultrasound of the abdomen showed hepatomegaly with a multinodular appearance and splenomegaly. Four blood samples were cultured on different days during his illness. Gram-negative colonies showed an API20NE biochemical profile of 1156576, suggesting *B. pseudomallei* (99% identity). After melioidosis was presumptively diagnosed, the patient was immediately treated with ceftazidime but he died 24 hours later, 2 weeks after admission.

DNA extracted from the presumptive *B. pseudomallei* isolates was positive by real-time PCR (*7*). Multilocus sequence typing (MLST) was performed as previously described (*8*). MLST demonstrated 2 novel allele combinations of 4, 12, 3, 2, 5, 2, 1 for case-patient 1 and 4, 12, 34, 1, 5, 2, 1 for case-patient 2. Sequences were submitted to the MLST database (http://bpseudomallei.mlst.net/) and have been assigned the sequence types (ST) 1053 and 1054, respectively.

Conclusions

The fatal outcome of these 2 case-patients reflects the reality of medical care prevailing in Madagascar and much of Africa, where hospital resources are limited and the capability for diagnosing melioidosis as part of routine laboratory practice does not exist. During 1936–2012, *B. pseudomallei* was isolated on rare occasions from Madagascar. In 1936, the Whitmore bacillus was isolated from a submaxillary node of a slaughtered pig by G. Girard, who was working at the Pasteur Institute in Madagascar. This

Figure. Map of Madagascar. Inset: Africa showing location of Madagascar.

was the first time this bacterium was identified in Africa (*9*). In 1982, M. Galimand and A. Dodin, working in the Whitmore bacillus laboratory at the Pasteur Institute in Paris, published a review of the distribution of *Pseudomonas pseudomallei* worldwide (*10*) and reported that in 1977 this bacterium was found in the soil of a zoo and pig farm in Antananarivo. In 2004, a French patient coming from Madagascar was diagnosed in La Réunion with melioidosis (*11*). He lived in Mahajanga city, but 25 years earlier had lived in Vietnam, where melioidosis is endemic. In 2005, melioidosis was diagnosed in a second French patient in La Réunion that was also attributed to infection in Madagascar; this person lived in Antananarivo, but disease onset occurred during a stay in Mahajanga (*12*). In 2013, melioidosis was diagnosed in Brussels in a third French patient living in Belgium who had returned from Mahajanga (*13*). His history did not reveal travel to any other melioidosis-endemic areas, and his infection was likely to have been acquired in Mahajanga.

Mahajanga has a tropical savanna climate; the rainy season is mostly from December to April. The 2 cases reported here occurred at the end of the rainy season; onset of all 3 published cases in French visitors occurred during March (2004, 2005, 2013). Before the 2 cases reported here from Madagascar, the only confirmed autochthonous case of melioidosis from Africa of which we were aware was in a 16-month-old boy from a remote village in rural Malawi who sought care in March 2011 (*3*). That case was also able to be diagnosed because of enhanced laboratory facilities associated with research support. In Mauritius, the first autochthonous confirmed case of melioidosis was diagnosed in 2004 (*4*).

Genotyping of the 2 *B. pseudomallei* strains reported here showed novel STs for both. Furthermore, the MLST result from the third French case-patient recently reported (*13*) demonstrated another novel allelic profile, 4, 1, 3, 2, 5, 2, 1, which has been designated ST1043. ST1043 is a single locus variant of ST1053 from case-patient 1 and ST1054 from case-patient 2 is a single locus variant of ST319, which is represented only by a single *B. pseudomallei* isolate cultured from the 2004 Mauritius case-patient (*4*). The B. pseudomallei isolate cultured from the recently reported Spanish traveler to Africa was ST879 (*2*), which is very divergent from STs 1043, 1053, and 1054, and suggests that her infection might have been acquired in 1 of the 14 West African countries she traveled through, rather than in Madagascar, which she also visited.

Although these genotype data are consistent with the findings recently reported from Africa that suggested a possible recent ancestor for strains from Malawi and Kenya, an alternative hypothesis is that the diversity and novel STs seen in Madagascar might represent more ancient origins for *B. pseudomallei* in Africa. Phylogeographic reconstruction of *B. pseudomallei* genomes has supported an Australian origin for *B. pseudomallei*, with a possible single introduction event into Southeast Asia, possibly during the last ice age when low sea levels created land bridges between what are now islands in the Malay Archipelago (*14*). Nevertheless, studies by Pearson et al. focused on strains from Australia and Southeast Asia and included few strains from the rest of the world. Without detracting from that hypothesis of spread from Australia to Southeast Asia, we suggest that a prior hypothesis of more ancient origins of *B. pseudomallei* (*15*) also could explain the novel and diverse genotypes of *B. pseudomallei* in Africa and in the Americas (*1*). Ongoing whole-genome sequencing of multiple isolates from diverse locations should resolve the origins and global dispersal patterns of *B. pseudomallei*.

In summary, 2 fatal cases of melioidosis in farmers who had diabetes confirm that melioidosis is endemic in Madagascar. Difficulties with diagnosis and treatment of melioidosis in less developed countries, such as Madagascar, highlight

the need for support for improved laboratory services and for further collaborative studies to elucidate the epidemiology of melioidosis in regions such as Africa where its presence is suspected but where data are very limited.

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Treatment of Giardiasis after Nonresponse to Nitroimidazole

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During January 2008–October 2013, a total of 12 cases of giardiasis at the Chaim Sheba and Shaare Zedek Medical Centers, Israel, did not respond to nitroimidazole; 83.3% were associated with travel and 33% with immunoglobulin deficiency. Among 110 published cases, the most effective treatment was quinacrine (efficacy 90%–100%), but its availability is limited.

Giardia lamblia are protozoan parasites distributed globally and mostly transmitted by close contact and consumption of contaminated water or food. Giardiasis occurs in industrialized nations (*1*); occasional waterborne outbreaks have been reported in North America and Europe (*2*,*3*). However, the prevalence of giardiasis is greater in developing countries, >50% among children from many locales (*4*). Among travelers, especially those traveling to developing countries, *G. lamblia* infection is also common; according to the GeoSentinel registry, it is the most frequently diagnosed gastrointestinal pathogen (*5*).

The utility of offering antigiardia medications to patients in areas of giardiasis hyperendemicity has been called into question unless symptoms are severe. Conversely, treatment administered to patients in areas of low giardiasis endemicity (such as ill returning travelers) usually leads to cure (*6*). Since the introduction of metronidazole in 1959, nitroimidazoles have been the main treatment for giardiasis. However, a recent meta-analysis of clinical trials has shown that nitroimidazole treatment fails for 10%–20% of giardiasis patients (*7*); optimal treatment in such cases is not defined, and some agents (e.g., quinacrine) are often unavailable.

The Study

During January 2008–October 2013, a total of 12 cases of nitroimidazole treatment failure (parasitologically confirmed by either microscopy or antigen testing of fecal samples after nitroimidazole therapy) were seen at the Center for Geographic Medicine and Tropical Diseases at Chaim Sheba Medical Center, Tel Hashomer, and the Tropical Disease Clinic at the Shaare Zedek Medical Center, Jerusalem, Israel. All patients were symptomatic.

Median patient age was 25.0 years (interquartile range 23.7–35.0, mean ± SD 30.4 ± 11.7 years), and 75% of patients were male (Table 1). Of the 12 cases, 10 (83.3%) occurred after travel to developing countries; 8 patients had traveled to Asia (India and/or Thailand) and 2 to Latin America. Of 11 patients evaluated for immune deficiency, 4 (36.4%) had low immunoglobulin levels; of these, 1 patient had pan-hypogammaglobulinemia and 3 had IgA deficiency.

The median number of failed courses of nitroimidazole was 2.5 (interquartile range 1–3, mean ± SD 2.7 ± 2.5 courses). All 12 patients received albendazole as a second line of treatment. Of 10 patients for whom complete parasitologic data were available, 4 (40%) experienced cure. Of 6 patients for whom albendazole failed, 4 received nitazoxanide. Of these, nitazoxanide led to cure for 1 (25%); subsequent treatment with quinacrine led to cure for 2, and treatment with paromomycin led to cure for the other.

Conclusions

Despite the high prevalence and global reach of giardiasis, reports of the treatment approach for cases that fail to respond to nitroimidazole are scarce. A review of the literature identified only 12 reports describing treatment outcomes for giardiasis patients after nitroimidazole treatment failure: 7 case series and 5 isolated case reports (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/10/14-0073-Techapp1.pdf). Together with the series reported here, 110 cases have been described (Table 2). Before 1994, only 2 (2%) cases had been reported; during 1994–2003 a total of 29 (26%) cases, and during 2004–2013 a total of 79 (72%) cases.

For the group of 110 patients, regimens combining (the already failed) nitroimidazoles with other agents led to a cure rate of 86.1% (Table 2). The most prominent effect of nitroimidazole combination was found for albendazole; monotherapy resulted in a cure rate of only 18.7%, whereas combination with nitroimidazole led to a cure rate of 80.8% (Fisher exact test $p<0.001$). Consistently, the best results were achieved with quinacrine; 90.5% and 100% of patients were cured with monotherapy and in combination with nitroimidazole, respectively. Only 2 (40%) of 5 and 5 (29.4%) of 17 patients who experienced nitroimidazole treatment failure were cured after treatment with nitazoxanide and paromomycin monotherapy, respectively.

Thus, most antiprotozoal agents seem to perform poorly after nitroimidazole has failed. Clearance of giardia after nitroimidazole treatment failure was achieved

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Table 1. Management of giardiasis nonresponsive to nitroimidazole treatment, Israel, January 2008–October 2013*

Patient age, y/sex	Travel history	No. failed nitroimidazole courses	Immunologic assessment	Alb	Nitaz	Quin	Paromo
24/M	Israel	9	Pan-hypogammaglobulinemia	Failed†	Failed	NA	NA
35/M	Latin America	3	IgA deficiency	Failed	Failed	Cured	NA
61/M	Israel	1	IgA deficiency	Cured	NA	NA	NA
23/M	Latin America	1	IgA deficiency	Cured‡	NA	NA	NA
25/M	Thailand	3	Normal	Failed	Failed	Cured	NA
45/M	India	3	Normal	Failed	Cured	NA	NA
31/F	India	2	Normal	Failed	NA	NA	Cured‡
26/M	India	1	Normal	Failed	NA	NA	Cured
23/M	India	1	Normal	Cured	NA	NA	NA
24/M	Thailand	1	Normal	Cured	NA	NA	NA
25/F	India	2	Normal	Cured‡	NA	NA	NA
23/F	India, Thailand	1	ND	Cured†	NA	NA	NA

*Alb, albendazole; NA, not administered; ND, not done; nitaz, nitazoxanide; paromo, paromomycin; quin, quinacrine.
†Combination albendazole–tinidazole treatment.
‡Patient was clinically asymptomatic but did not provide a posttreatment fecal sample.

for <20% of patients who received albendazole and <30% of patients who received paromomycin, despite reported cure rates of 80% (*7*) and 90% (*8*), respectively, for these drugs when used as primary regimens. Clinical trials have reported that the giardia clearance rate after primary monotherapy with nitazoxanide also approached 80% (*9*). Its role in cure after nitroimidazole treatment failure has not been established, but in the few cases found in our search, the giardiasis cure rate after nitazoxanide treatment was only 40% (Table 2). Thus, nitroimidazole treatment failure might actually be an indicator for multidrug-resistant *G. lamblia* strains.

Whether nitroimidazole treatment failure reflects pathogen resistance or defective host defenses is not clear. In our case series and others, immunoglobulin deficiency was common in cases of nitroimidazole treatment failure. It has been shown in select cases that nitroimidazole therapy in immunoglobulin-deficient patients fails to clear nitroimidazole-sensitive giardia, leading to in vivo emergence of resistant strains (*10*). The role of a host factor might also be evidenced by cases in which giardia are refractory to nitroimidazole in 1 patient but easily eliminated from the patient's family members, as occurred for a patient in our series. This 35-year-old male patient was IgA deficient, and his wife and toddler son were asymptomatic carriers of giardia, according to fecal testing; giardiasis was parasitologically cured by metronidazole for the family members but not for the patient, for whom repeated courses of the same treatment and other regimens failed. Similar reports from other case series include a family of 4 who were infected simultaneously with *G. lamblia* while traveling in India; although

Table 2. Reports on management of giardiasis nonresponsive to nitroimidazole treatment worldwide, 1962–2013*

Country and publication year	No. cases	No. patients who visited developing countries	No. cured/no. treated (% cured), by treatment type						
			Alb	Paromo	Nitaz	Alb + nitroimid	Quin	Quin + nitroimid	Paromo + nitroimid
Israel, 2014 (this study)	12	10	3/9 (33.3)	1/1 (100)	1/4 (25.0)	1/2 (50.0)	2/2 (100)	–	–
Spain, 2014	3	3	–	–	–	–	3/3 (100.0)	–	–
Spain, 2013	14	14	0/2	0/4	–	–	14/14 (100)	–	–
Spain, 2010	10	8	0/2	0/3	–	1/1 (100)	–	4/4 (100)	2/2 (100)
Norway, 2008	38	0	–	3/6 (50.0)	–	30/38 (78.9)	–	3/3 (100)	–
United States, 2001	5	0	0/2	1/3 (33.3)†	–	–	–	5/5 (100)	–
France, 2000	3	0	1/3 (33.3)	–	–	–	–	–	–
Italy, 1995	20	0	2/10 (20.0)	–	–	9/10 (90.0)	–	–	–
Single case reports, 1962–2008‡	5	1	0/4	–	1/1 (100)	1/2 (50.0)	0/2	2/2 (100)	–
Total	110	36	6/32 (18.7)	5/17 (29.4)	2/5 (40.0)	42/53 (79.2)§	19/21 (90.5)	14/14 (100)	2/2 (100)

*Complete reference information available in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/10/14-0073-Techapp1.pdf). Alb, albendazole; nitaz, nitazoxanide; nitroimid, nitroimidazole; paromo, paromomycin; quin, quinacrine; –, drug not used.
†Used in combination with bacitracin.
‡Single case reports from France, Switzerland, Canada, Saudi Arabia, and Thailand.
§p<0.001 by Fisher exact test for comparison with alb monotherapy.

PCR indicated that the strains from all 4 patients were genetically identical, responses to treatment with tinidazole varied (*11*).

Given the poor performance of other monotherapies, the 90.5%–100% rate of response to quinacrine is remarkable. Shortly after the introduction of metronidazole, randomized trials showed it to be as efficacious as quinacrine (*12*), and metronidazole replaced quinacrine entirely as a giardiasis treatment. After 3 decades, the situation seems to be changing; nitroimidazole treatment failure is increasing, and quinacrine seems to be the best treatment for giardiasis.

It is unfortunate that quinacrine is no longer available through pharmaceutical companies; in some countries, it can be obtained only through compounding pharmacies or not at all. In Israel, for example, quinacrine is practically unavailable; for 1 patient in our series, quinacrine was obtained with the kind assistance of colleagues practicing in a developing country. This case illustrates how pharmaceutical industry neglect of tropical parasitic infections carries a health price tag, even in industrialized countries.

Several sources of bias may pertain to retrospective case series, the only extant source of data on nitroimidazole treatment failure for giardiasis. Case series are sometimes the result of point-source (often waterborne) outbreaks and therefore might be biased by the presence of 1 or a few pathogen strains (*3*). Even in reports in which cohorts are more geographically diverse, numbers of patients are generally small and statistical comparison of treatment outcomes is not possible. Moreover, the choice of antiprotozoal agents is influenced by factors other than effectiveness, such as drug availability and cost, which differ from country to country. However, combining all reported cases as we have done eliminates geographic bias, increases numbers, and makes it possible to offer a more reliable view of the treatment of giardiasis not responsive to nitroimidazole.

Among giardiasis patients, nitroimidazole treatment failure is often associated with failure of antiprotozoal drugs in additional classes and with patient immunoglobulin deficiency. Limited data exist to guide treatment when nitroimidazole fails. However, this review of reported cases suggests for this scenario, quinacrine is highly effective and nitroimidazole–albendazole combination therapy is far superior to albendazole monotherapy. Unfortunately, quinacrine is unavailable in many countries, leaving patients with limited and less reliable therapeutic options.

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Human Infection with Highly Pathogenic A(H7N7) Avian **Influenza Virus,** Italy, 2013

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During an influenza A(H7N7) virus outbreak among poultry in Italy during August–September 2013, infection with a highly pathogenic A(H7N7) avian influenza virus was diagnosed for 3 poultry workers with conjunctivitis. Genetic analyses revealed that the viruses from the humans were closely related to those from chickens on affected farms.

In Europe, avian influenza viruses of subtype H7 have been responsible for several disease outbreaks among poultry, which resulted in human infections (*1*,*2*). Notably, since 2000, outbreaks of avian influenza caused by high and low pathogenicity influenza A(H7N1) viruses and low pathogenicity A(H7N3) viruses occurred on poultry farms located mainly in northeastern Italy (*3*). On August 14, 2013, infection caused by a highly pathogenic avian influenza A(H7N7) virus was initially detected on a layer farm in Ostellato, Ferrara Province, Italy, representing the start of an epizootic that affected another 5 poultry farms in Ferrara and Bologna Provinces (Emilia-Romagna Region) during the next 3 weeks. Nearly 1 million chickens on the 6 farms were culled (*4*). All workers (≈200) who participated in depopulating infected premises applied strict infection prevention procedures and were monitored for symptoms. Among the workers, infection with highly pathogenic A(H7N7) avian influenza virus was confirmed for 3 who had conjunctivitis but no respiratory symptoms. We describe the clinical and virologic findings of the investigation conducted with regard to these 3 human cases of influenza A(H7N7) virus infection.

The Study

On August 28, 2013, a previously healthy 51-year-old poultry worker (patient 1) noted unilateral conjunctivitis. After the worker was examined by a physician, a conjunctival swab sample was collected and tested at St. Orsola Hospital in Bologna; it was positive for influenza A virus subtype H7. Three days later, a 46-year-old poultry worker (patient 2) sought care for bilateral conjunctivitis and other symptoms, such as chills and muscle aches. On September 4, a 49-year-old man (patient 3) sought care for bilateral conjunctivitis. Conjunctival swab samples collected from patients 2 and 3, tested at the same laboratory, also produced positive results for influenza A virus subtype H7.

Patients 1 and 2 worked with breeding and cleaning on a farm in Mordano, Bologna Province; they had not used personal protective equipment (PPE) until August 21, when influenza A(H7N7) virus infection in poultry was diagnosed. Thereafter, they were involved in culling and wore PPE, including face masks with eye protection. Patient 3 had not previously worked with animals, but he participated in depopulation procedures, while wearing PPE, during the 3-week outbreaks on the farms in Ostellato and Mordano. Because the 3 patients had worked for the same poultry company on the affected farms, located inside a 1.5-km–radius area, the date of exposure for each patient is difficult to infer (*4*). All 3 patients were isolated at home; without specific antiviral treatment, symptoms resolved in a few days. Six family contacts were placed under clinical surveillance for 10 days.

Conjunctival swab samples were collected from each patient, and aliquots of these samples were sent to the Istituto Superiore di Sanità, Rome, Italy, where they were confirmed as influenza virus subtype H7N7 by 2 real-time reverse transcription PCR (rRT-PCR) assays (*5*,*6*). A traditional RT-PCR assay was conducted by using specific

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primers (available upon request), and PCR products were sequenced by using a BigDye Terminator version 3.1 kit (Life Technologies, Austin, TX, USA). Full-genome sequencing was also performed by using the Ion Torrent PGM apparatus (Life Technologies, Carlsbad, CA, USA). Virus isolation in MDCK cells was successful for patient 3 only, whose conjunctival swab sample showed the highest virus load as revealed by a low cycle threshold (18.3) obtained by rRT-PCR (*5*). Full-length gene sequencing was conducted on the clinical sample from patient 3 (GenBank accession nos. KF918334–KF918341), whereas the sequence analysis of the other 2 samples was focused on hemagglutinin and neuraminidase genes and a few regions of the other genes (nt 159–525 and nt 994–1107 of polymerase basic protein [PB] 2, nt 115–453 and nt 1726–2259 of PB1, nt 19–297 of nucleocapsid protein, and nt 715–981 of matrix protein) to trace the avian virus responsible for the human infections. In particular, the virus from the chicken with the index case (A/chicken/Italy/13VIR4527–11/2013) was isolated on August 13 in Ostellato, and 6 other strains were isolated from chickens during the 6 outbreaks that occurred among poultry until September 3, 2013 (*7*) (Table 1). Antigenic characterization of the subtype H7N7 virus isolated from a human was conducted by hemagglutination inhibition assay with turkey erythrocytes (*8*) and H7 reference antiserum. To define virus susceptibility to neuraminidase inhibitors (oseltamivir and zanamivir), we performed the fluorescent MUNANA (2′-[4-methylumbelliferyl]-±-D-N-acetylneuraminic acid)–based assay (*8*).

The 3 patients were infected with an avian-origin influenza A(H7N7) virus. In particular, molecular analyses showed that the hemagglutinin and neuraminidase gene sequences of A/Italy/3/2013 virus isolate (GenBank accession nos. KF712391, KJ136817) were identical to those of the clinical specimens. The hemagglutinin amino acid sequence of this virus, and those from patients 1 and 2, showed complete homology to most of the isolates from chickens, including the hemagglutinin cleavage site containing multiple basic amino acids (PKRRERR*GL) responsible for the highly

Table 1. Comparison of the nucleotide sequences of highly pathogenic avian influenza A(H7N7) virus isolate from chickens and humans, Italy, August–September 2013*

Virus, location	PB2				PB1			PA	HA			NP	NA				M	
(date)†	232‡	279	333	1044	183‡	1882	2133	1251	471	1018‡	1410	72	515‡	541‡	1040‡	1347	818‡	884‡
Chicken																		
A/CK/4527, Ostellato (Aug 13)§	C	T	T	C	C	C	C	G	A	G	G	G	A	T	C	A	A	G
A/CK/4541, Ostellato (Aug 13)	T	•	•	•	•	•	T	•	G	A	•	•	•	A	•	•	G	•
A/CK/4603, Mordano (Aug 19)	T	•	•	•	•	•	T	•	•	•	•	•	•	A	•	G	G	•
A/CK/4678, Portom (Aug 21)	T	•	•	T	•	•	T	•	•	•	•	A	•	A	•	G	G	•
A/CK/4774, Mordano (Aug 27)	T	•	C	•	•	T	T	•	•	•	A	•	•	A	•	•	•	•
A/CK/5091, Bondeno (Sep 2)	T	C	•	T	T	•	T	•	•	•	•	A	•	A	•	G	G	A
A/CK/5051, Mordano (Sep 3)	T	•	•	–	•	•	T	A	•	•	•	•	•	A	•	•	•	•
Human¶																		
1, Mordano (Aug 29)	T	•	•	•	•	•	T	–	•	•	•	•	•	A	•	–	G	•
2, Mordano (Sep 2)	T	•	•	•	•	•	T	–	•	•	•	•	•	A	•	•	•	•
3, Mordano /Ostellato (Sep 7)	T	•	•	•	•	•	T	A	•	•	•	•	G#	A	A#	•	•	•

*Dots indicate no nucleotide changes; –, notavailable. HA, hemagglutinin; NA, neuraminidase; NP, nucleocapsid protein; M, matrix; PA, polymerase acidic; PB, polymerase basic; Portom, Portomaggiore.
†Date indicates date collected. A/CK/4527, A/chicken/Italy/13VIR4527–11/2013; A/CK/4541, A/chicken/Italy/13VIR4541–34/2013; A/CK/4603, A/chicken/Italy/13VIR4603/2013; A/CK/4678, A/chicken/Italy/13VIR4678–1/2013; A/CK/4774, A/chicken/Italy/13VIR4774/2013; A/CK/5091, A/chicken/Italy/13VIR5091–1/2013; A/CK/5051, A/chicken/Italy/13VIR5051–3/2013.
‡Nonsynonymous substitution.
§First isolate from chicken (index case).
¶Clinical samples.
#Nucleotide changes found only in the specimen from patient 3 and responsible for the D172G and P347Q amino acid changes.

Table 2. Antigenic analyses of influenza A/H7 viruses*

Viruses	Antiserum A/Anhui/1/13 (ferret)	A/TK/It/3889/99 (ferret)†	A/TK/It/214845/02 (ferret)	A/GS/CZ/1848/09 (chicken)‡	A/TK/Eng/647/77 (chicken)‡	A/AS/Eng/983/79 (chicken)‡
Reference						
A/Anhui/1/2013 (H7N9)	**320**	80	80	80	40	80
A/turkey/Italy/3889/1999 (H7N1)	80	**80**	80	40	<40	<40
A/turkey/Italy/214845/2002 (H7N3)	40	80	**80**	<40	<40	40
A/goose/Czech Republic /1848/2009 (H7N9)§	40	40	40	**320**	40	40
A/turkey/England/647/1977 (H7N7)	40	<40	40	40	**160**	40
A/African starling/England/983/1979 (H7N1)§	40	<40	40	40	40	**160**
Test						
A/Italy/3/2013 (H7N7)	40	40	<40	160	40	80

*Data are hemagglutination inhibition titers. Homologous titers of reference viruses to serum samples are indicated in boldface. AS, African starling; GS, goose; TK, turkey.
†Antiserum from National Institute for Biologic Standards and Control, UK.
‡Antiserum from Animal Health and Veterinary Laboratories Agency, UK.
§Inactivated virus.

pathogenic phenotype (*9*). The neuraminidase sequence from the isolate from patient 3 differed from those of clinical specimens from the other 2 patients and most isolates from chickens by 2 amino acids (D172G and P347Q) (Table 1). Neither neuraminidase stalk deletions nor neuraminidase inhibitor sequence-based resistance were detected in the influenza viruses from the humans (*10*,*11*); the drug sensitivity of the viruses was further confirmed by phenotypic neuraminidase inhibitor susceptibility assays performed on A/Italy/3/2013 virus (data not shown).

Phylogenetic analyses of the hemagglutinin and neuraminidase genes (Figures 1, 2) confirmed that the H7N7 isolates from the human patient in Italy were closely related to H7 low pathogenicity avian influenza viruses that had been circulating among wild birds and poultry in Europe during the past 3 years. Antigenic characterization of the A/Italy/3/2013 virus by hemagglutination inhibition assay (Table 2) showed that the virus was recognized poorly by antiserum raised against A/Anhui/1/2013 (H7N9), A/turkey/Italy/214845/2002 (H7N3), and A/turkey/England/

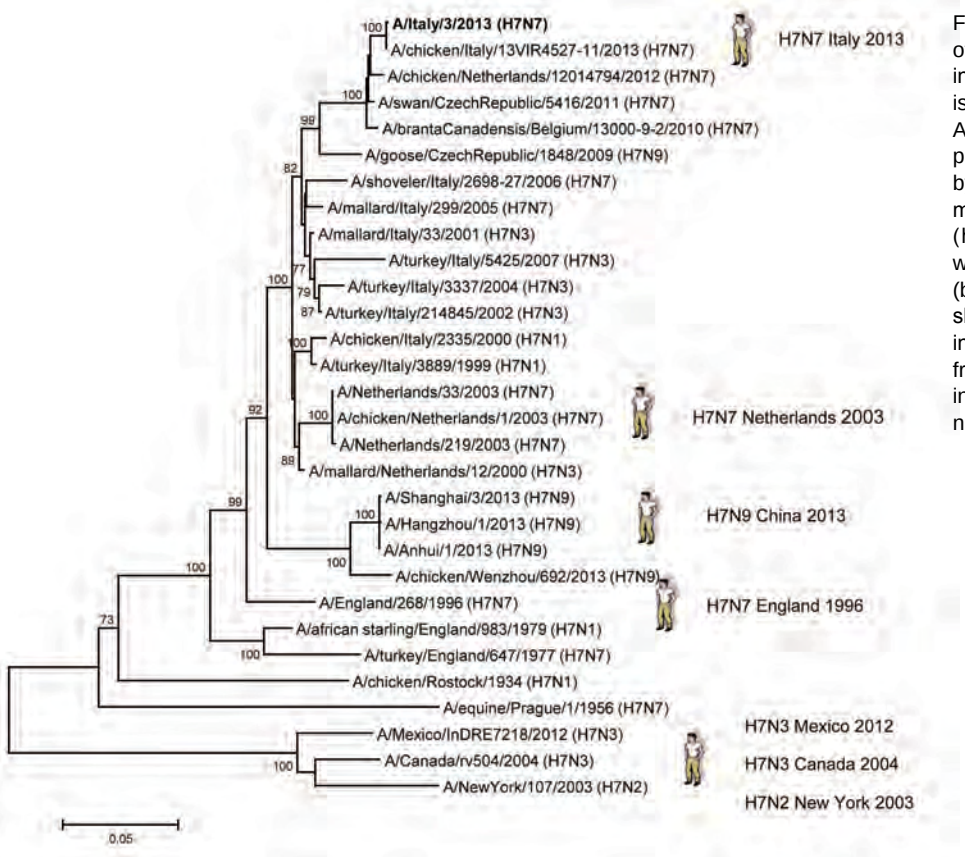

Figure 1. Phylogenetic analysis of the hemagglutinin gene of the influenza A(H7N7) virus, Italy, isolated from humans during August–September 2013. The phylogenetic tree was constructed by using the neighbor-joining method and MEGA 5 software (http://www.megasoftware.net) with 1,000 bootstrap replicates (bootstrap values ≥70% are shown next to nodes). The influenza A(H7N7) virus isolated from a human in 2013 is shown in boldface. Scale bar indicate nucleotide substitutions per site.

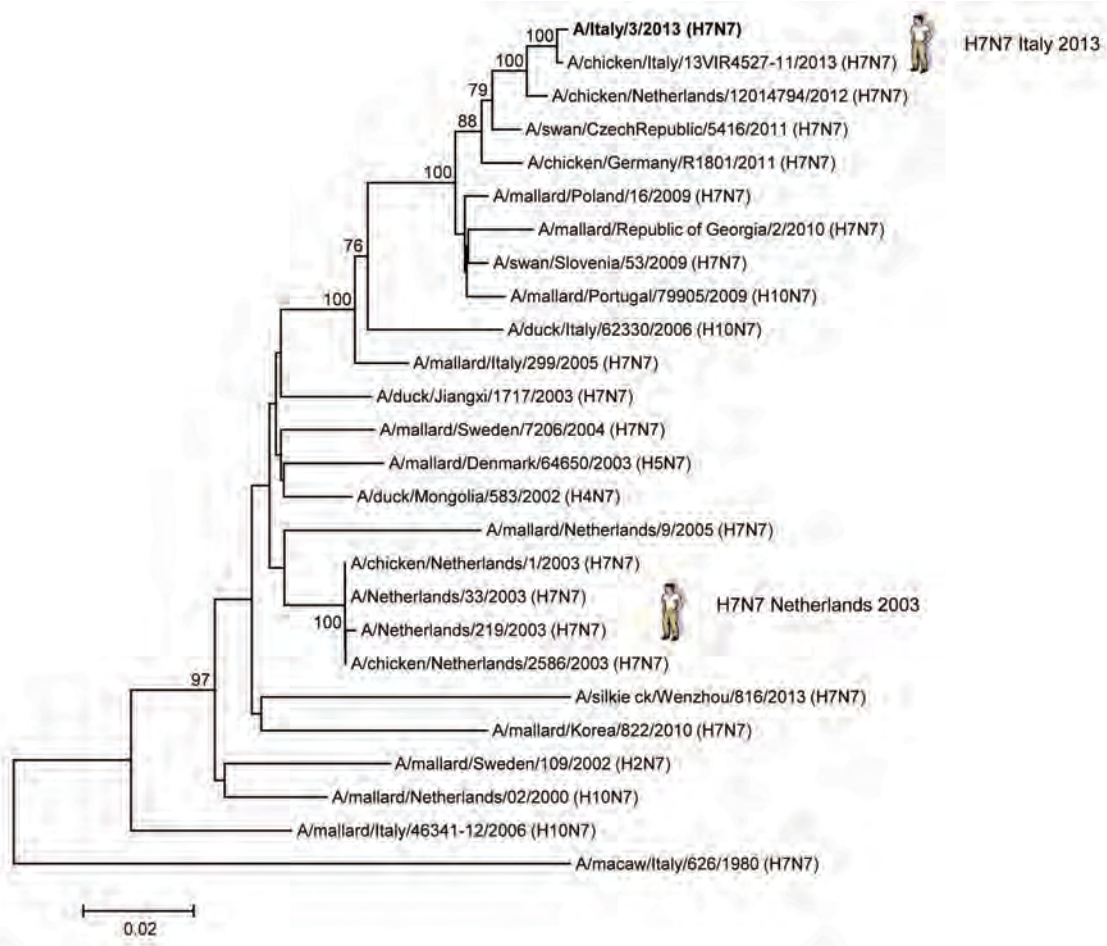

Figure 2. Phylogenetic analysis of the neuraminidase gene of the influenza A(H7N7) virus, Italy, isolated from humans during August–September 2013. The phylogenetic tree was constructed by using the neighbor-joining method and MEGA 5 software (http://www.megasoftware.net) with 1,000 bootstrap replicates (bootstrap values ≥70% are shown next to nodes). The influenza A(H7N7) virus isolated from a human in 2013 is shown in boldface. Scale bar indicate nucleotide substitution per site.

647/1977 (H7N7) and recognized well by antiserum raised against A/goose/Czech Republic/1848/2009 (H7N9), A/African starling/England/983/79 (H7N1), and A/turkey/Italy/3889/99 (H7N1).

Genome sequences of the internal proteins of A/Italy/3/2013 virus and from the clinical samples revealed high identity to the circulating H7N7 strains from chickens in the area. Although deducing the exact exposure for each patient is difficult, partial sequence analysis showed that the virus from patient 1 was more related to A/chicken/Italy/13VIR4603/2013 and that the viruses from patients 2 and 3 were more related to A/chicken/Italy/13VIR5051–3/2013 (Table 1). These 2 viruses from chickens had been isolated during different outbreaks in Mordano (*7*) and differed somewhat from that from the chicken with the index case (A/chicken/Italy/13VIR4527–11/2013). Neither mammalian host adaptation markers, including the E627K mutation in PB2, nor the common mutations associated with adamantane resistance (L26F, V27A/G, A30S/T/V, S31N, G34E) in matrix protein 2 were found in the H7N7 strain from humans (*12–14*).

Conclusions

This study provides further evidence of H7 subtype–specific ocular tropism (*1*). Our molecular findings suggest direct transmission of the virus from chickens to humans; the lack of known host adaptation markers does not support human-to-human transmission. The presence of 2 mutations in neuraminidase from the specimen of patient 3, which contained the highest viral load, might suggest a correlation with the efficiency of infection and replication in the conjunctiva. Indeed, specific neuraminidase mutations have been observed in H7N7 viruses from the Netherlands and have been associated with enhanced replication (*15*). However, further studies are needed to

determine their exact role in the pathogenesis of the infection. Clinical surveillance was immediately applied to all exposed workers and cohabiting contacts, and no further human cases of H7N7 infection were identified. For the purpose of investigating human subclinical infections by H7 viruses, a serologic surveillance program is ongoing in the affected areas.

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Rickettsia parkeri and *Rickettsia montanensis*, Kentucky and Tennessee, USA

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We found that 14.3% (15/105) of *Amblyomma maculatum* and 3.3% (10/299) of *Dermacentor variabilis* ticks collected at 3 high-use military training sites in west-central Kentucky and northern Tennessee, USA, were infected with *Rickettsia parkeri* and *Rickettsia montanensis,* respectively. These findings warrant regional increased public health awareness for rickettsial pathogens and disease.

The Gulf Coast tick (*Amblyomma maculatum*) has become well-established in states outside its historically described coastal range, most recently in North Carolina and Virginia (*1*,*2*). This tick has been sporadically reported in other states, including Tennessee and Kentucky (*3*,*4*). *A. maculatum* ticks are the recognized vector of *Rickettsia parkeri*, a spotted fever group (SFG) bacterium that is pathogenic to humans and has caused illness in ≥32 patients (*5*–*7*; C. Paddock, unpub. data).

R. parkeri–infected *A. maculatum* ticks from Kentucky were among specimens submitted to the human tick–testing program of the US Army during 2000–2009 (*4*), which increased concern of a potential health threat to military personnel using field training areas. To assess the threat of human exposure to *R. parkeri* and other potential rickettsial pathogens, we conducted a tick survey at 3 high-use military training sites in west-central Kentucky and northern Tennessee, USA.

The Study

Questing ticks were collected during July 16–20, 2012, by using cloth drags, flags, and CO_2-baited traps, and by removing ticks from collectors (Table 1). Sites of collection were Fort Campbell (Christian County, Kentucky, and Montgomery County, Tennessee), Fort Knox (Bullitt, Hardin, and Meade Counties, Kentucky), and Wendell H. Ford Regional Training Center (WHFRTC; Muhlenberg County, Kentucky).

Multiple 2-person teams collected ticks during 15-minute periods; an average of 19 person-hours was spent sampling at each site. Target tick species were *A. maculatum* and *Dermacentor variabilis*, although *A. americanum* ticks were also collected. Human encounter rates (calculated by using all collection methods except CO_2-baited traps) for adult *A. maculatum* and *D. variabilis* ticks were ≈2 ticks/hour and 5 ticks/hour, respectively. No immature stages of these species were encountered. Field sites sampled were dominated by sericea (*Lespedeza cuneata*) and fescue (*Festuca pratensis*). Some adjacent areas had switchgrass (*Panicum virgatum*) and Indiangrass (*Sorghastrum nutans*). *A. maculatum* ticks appeared tolerant of exposed, unshaded sites and were often collected in the middle of these fields.

Ticks were identified by using the key of Keirans and Litwak (*8*). Specimens were individually placed in microcentrifuge tubes containing 500 μL of tissue lysis buffer (QIAGEN, Valencia, CA, USA) and 20 μL of proteinase K (QIAGEN), bisected with a sterile blade, and incubated at 56°C for ≥1 h. Nucleic acid was extracted by using the DNeasy Blood and Tissue Kit (QIAGEN).

Initial quantitative real-time PCRs (qPCRs) were performed by using the *Rickettsia*-specific Rick17b assay specific for the 17-kD antigen gene (*4*) and the LightCycler TaqMan Master (Roche Applied Sciences, Indianapolis, IN, USA) ready-to-use hot start reaction mixture in the LightCycler 2.0 instrument (Roche Applied Sciences). Final reactions contained 5 μL of template and 15 μL of master mixture. Master mixture contained 0.5 μmol/L primers, 0.4 μmol/L probe, LightCycler TaqMan Reaction Mixture (Roche Applied Sciences), and water. All qPCRs were performed at 95°C for 10 min and for 45 cycles at 95°C for 15 s and 60°C for 30 s.

Positive samples were further evaluated by using the SFG *Rickettsia*-specific conventional PCR with primer pair Rr190.70p and Rr190.602n, which is specific for the outer membrane protein A (*ompA*) gene of *Rickettsia* spp. and speciated by using *Pst*I restriction fragment length polymorphism analysis (*9*). Identities of 9 positive samples were confirmed by sequencing a fragment of *ompA* (1,651 bp) or *ompB* (1,540 bp) genes (Table 2) (*10*). All *A. maculatum* tick samples positive for *Rickettsia* spp. were also tested for *Candidatus* Rickettsia andeanae by using the Rande qPCR (*4*).

A total of 404 adult ticks (105 *A. maculatum* and 299 *D. variabilis*) were collected and tested. Of these ticks, 3 *A. maculatum* and 44 *D. variabilis* ticks were collected from Fort Knox, 66 *A. maculatum* and 148 *D. variabilis* ticks were collected from Fort Campbell, and 36 *A. maculatum* and 107 *D. variabilis* ticks were collected from WHFRTC.

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Table 1. Quantitative PCR results for rickettsia in *Amblyomma maculatum* and *Dermacentor variabilis* ticks, Kentucky and Tennessee, USA, 2012

Location, tick species	No.	No. (%) positive for *Rickettsia parkeri*	No. (%) positive for *Rickettsia montanenesis*
Fort Knox, Kentucky			
A. maculatum	3	0	0
D. variabilis	44	0	2 (5)
Fort Campbell, Kentucky and Tennessee			
A. maculatum	66	10 (15)	0
D. variabilis	148	0	6 (4)
Wendell Ford Regional Training Center, Kentucky			
A. maculatum	36	5 (14)	0
D. variabilis	107	0	2 (2)
Total			
A. maculatum	105	15 (14)	0
D. variablilis	299	0	10 (3)

Twenty-five (6.2%) of 404 ticks were infected with an SFG *Rickettsia* species. *R. parkeri* was detected in 15 (14.3%) of the *A. maculatum* ticks.

The *ompA* sequences (GenBank accession no. KJ741849) of *A. maculatum* ticks collected from Fort Campbell (n = 2) and WHFRTC (n = 2) were identical to those of *R. parkeri* strain Portsmouth (GenBank accession no. CP003341) and *R. parkeri* Maculatum 20 (GenBank accession no. U83449). *R. montanensis* was detected in 10 (3.3%) of the *D. variabilis* ticks; isolates from 5 tick samples were sequenced. The *ompA* sequences (GenBank accession no. KJ741850) of *D. variabilis* ticks from Fort Knox (n = 1) and WHFRTC (n = 2) were 99.9% identical with *R. montanensis* str. OSU 85–930 (GenBank accession no. CP003340). The *ompB* sequences (GenBank accession no. KJ741851) of 2 *D. variabilis* ticks collected at Fort Campbell were 99.9% identical with those of *R. montanensis* str. OSU 85–930 (GenBank accession no. CP003340). No other *Rickettsia* spp., including *R. rickettsii*, were detected in any of the 404 ticks tested. The greatest percentage (15%) of *R. parkeri*–positive *A. maculatum* ticks were from Fort Campbell. *R. parkeri* was not detected in any of the *A. maculatum* ticks from Fort Knox.

Conclusions

Given that *A. maculatum* ticks were collected at multiple sites during multiple years, and that these ticks have recently been collected in large numbers, this species is probably established in west-central Kentucky and northern Tennessee. To further elucidate its distribution throughout both states, efforts should be made to collect immature stages of *A. maculatum* ticks from hosts, particularly birds.

The etiologic agent of Rocky Mountain spotted fever (RMSF), *R. rickettsii*, was not found in any of the ticks analyzed during this study, a finding that is consistent with findings of Fritzen et al. (*11*). However, during 2008–2012, a total of 15 human RMSF cases (5-year average rate of 0.1 cases/100,000 population) were reported to the Kentucky Department of Public Health (*12*). Likewise, for the same period, 1,695 cases of RMSF were reported to the Tennessee Department of Health (5-year average of 393 cases/100,000 population) (*13*). In addition, an *R. parkeri* human infection in Kentucky has been confirmed by PCR analysis of a tissue biopsy specimen from a patient (*5*). Thus, persons in west-central Kentucky and northern Tennessee may be more likely to become infected with a rickettsial agent other than *R. rickettsii*.

The tick encounter rates during this study suggest that persons entering appropriate habitats, especially for an extended period, are likely to encounter *D. variabilis* and *A. maculatum* ticks in west-central Kentucky and northern Tennessee during mid-summer. This study further suggests that although a person is ≈2.5 times more likely to encounter *D. variabilis* ticks than *A. maculatum* ticks, persons are ≈4.5

Table 2. Primers used for PCR, nested PCR, and sequencing for *Rickettsia parkeri* and *Rickettsia montanensis*, Kentucky and Tennessee, USA, 2012*

Gene, primer	Sequence (5′→3′)	Fragment, bp
ompB		
120-M59	CCGCAGGGTTGGTAACTGC	
ompB1570R	TCGCCGGTAATTRTAGCACT	PCR: 1,540
120–607F	AATATCGCTGACGGTCAAGGT	
120–807R	CCTTTTAGATTACCGCCTAA	
ompA		
190–3588F	AACAGTGAATGTAGGAGCAG	
RompA3182R	TTGCTGAGCGAAAYACTTACTYC	PCR: 3,202
190–5238R	ACTATTAAAGGCTAGGCTATT	Nested PCR: 1,651
RhoA4336F	AGTTCAGGAAACGACCGTA	
RompA4433R	TTTCCTGCAGTTACAGAATTTAAT	

**omp*, outer membrane protein.

times more likely to encounter an *R. parkeri*–positive *A. maculatum* tick than a rickettsia-positive *D. variabilis* tick. These results are consistent with those of Stromdahl et al. (*14*).

Further evidence is needed to confirm if *R. montanensis* in *D. variabilis* ticks is of medical concern, but there has been 1 report of tick-borne *R. montanensis* infection associated with a nonfebrile episode in a person with a rash (*15*). Because of the lack of awareness regarding *R. montanensis* infection, it is plausible that a rash could be misdiagnosed and assumed to be a sign of a different illness. Even if an illness was recognized as a vectorborne disease, rickettsial serologic assays are not able to distinguish 1 species of SFG rickettsia from another (*14*). This finding indicates that serologic reactivity caused by exposure to *R. montanensis* could be attributed to the wrong SFG rickettsiae. Other epidemiologic studies are needed to elucidate how these findings may relate to regional rickettsial illness, but they still confirm that *A. maculatum* ticks infected with *R. parkeri* and *D. variabilis* ticks infected with *R. montanensis* warrant increased public health awareness in this region.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Pandemic Fear and Literature: Observations from Jack London's The Scarlet Plague

Michele Augusto Riva,[1] Marta Benedetti,[1] and Giancarlo Cesana

The Scarlet Plague, originally published by Jack London in 1912, was one of the first examples of a postapocalyptic fiction novel in modern literature (*1*). Set in a ravaged and wild America, the story takes place in 2073, sixty years after the spread of the Red Death, an uncontrollable epidemic that depopulated and nearly destroyed the world in 2013. One of the few survivors, James Howard Smith, alias "Granser," tells his incredulous and near-savage grandsons how the pandemic spread in the world and about the reactions of the people to contagion and death. Even though it was published more than a century ago, The Scarlet Plague feels contemporary because it allows modern readers to reflect on the worldwide fear of pandemics, a fear that remains very much alive.

By exploring the motif of the plague, a consistent and well-spread topos (i.e., theme) in literature (2–4), London's novel is part of a long literary tradition, inviting the reader to reflect on the ancestral fear of humans toward infectious diseases. In the ancient world, plague and pestilence were rather frequent calamities, and ordinary people were likely to have witnessed or heard vivid and scary reports about their terrible ravages (5). When plague spread, no medicine could help, and no one could stop it from striking; the only way to escape was to avoid contact with infected persons and contaminated objects (6). The immense fright was also fueled by a belief in the supernatural origin of pandemics, which were often believed to be provoked by offenses against divinities. In the Bible (e.g., Exodus 9:14, Numbers 11:33, 1 Samuel 4:8, Psalms 89:23, Isaiah 9:13), the plague was viewed as one of God's punishments for sins, so the frightening description of its spread was interpreted as a warning to the Israelites to behave morally. This causal relationship between plague and sin is seen also in Greek literary texts, such as Homer's Iliad and Sophocles' Oedipus the King (429 BCE).

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In contrast, the Greek historian Thucydides (c. 460–395 BCE), in his History of the Peloponnesian War, and the Latin poet Lucretius (c. 99–55 BCE), in his De Rerum Natura, refuted a supernatural origin of the disease and focused their descriptions on the uncontrolled fear of contagion among the public. According to these authors, plague did not discriminate between the good and the evil but brought about the loss of all social conventions and a rise in selfishness and avarice.

Later medieval writings, such as The Decameron by Giovanni Boccaccio (1313–1375) and The Canterbury Tales by Geoffrey Chaucer (1343–1400), emphasized human behavior: the fear of contagion increased vices such as avarice, greed, and corruption, which paradoxically led to infection and thus to both moral and physical death (*7*,*8*). Human reactions to the plague are also the central themes of historical titles such as A Journal of the Plague Year by Daniel Defoe (1659–1731), a long, detailed narrative of events, anecdotes, and statistics regarding the Great Plague of London of 1665. In a similar manner, The Betrothed and History of the Column of Infamy, both written by Italian novelist Alessandro Manzoni (1785–1873), were extraordinary descriptions of the plague that struck Milan around 1630 (*9*).

In English-language literature, The Last Man (1826) by English novelist Mary Shelley (1797–1851) was one of the first apocalyptic novels, telling of a future world that had been ravaged by a plague; a few persons appear to be immune and avoid contact with others. The concept of immunization in this book demonstrates that the author, most famous for the novel Frankenstein, had a deep understanding of contemporaneous theories about the nature of contagion. In 1842, the American poet and novelist Edgar Allan Poe (1809–1849) published The Masque of the Red Death, a short story unique in the literary tradition of the plague by focusing only on the metaphorical element of the topos. Through the personification of the plague, represented by a mysterious figure disguised as a Red Death victim, the author meditates on the inevitability of death; the issue is not that people die from the plague, but that people are plagued by death (*9*).

[1]These authors contributed equally to this article.

The Scarlet Plague and the Fear of Pandemic

Jack London (1876–1916) was a US writer and journalist and author of classic novels including The Call of the Wild (1903) and White Fang (1906). He was also an active member of the Socialist Party of America, and his works often contained explicit critiques against capitalism and war. Numerous stories London wrote would today be classified as science fiction, and some had pandemics and infectious diseases as subjects. The Unparalleled Invasion (1910) described a biological warfare campaign launched from the United States and the other Western countries to arrest the uncontrolled growth of China's population and protect European colonies in Asia from Chinese immigration. In The Scarlet Plague, London investigated many traditional issues of the literary topos of plague, ranging from a reflection on morality and justice to the contagion and clinical features of the disease. In particular, the author focused his attention on behavioral responses to a pandemic, showing the emergence of fear, irrationality, and selfishness in a previously civilized and modern society. This novel differed greatly from earlier writings related to plague because it reflected deeply the contemporary scientific discoveries on pathogens fostered by scientists such as Louis Pasteur (1822–1895) and Robert Koch (1843–1910). By the early 20th century, epidemics were no longer considered divine punishments or supernatural events; 19th century bacteriologists had demonstrated that they are caused by germs that infect humans, and epidemiologists and public health experts had shed light on the mechanisms of disease transmission, including suggestions of general preventive measures to limit pandemics (*10*). Despite these scientific developments, however, in London's time, the general public's fear of the invisible world of microorganisms was still high.

In the novel, at the beginning of the epidemic of Scarlet Death, the people appeared not to be alarmed because they "were sure that the bacteriologists would find a way to overcome this new germ, just as they had overcome other germs in the past" (*1*). Public trust in science was high in the 21st century society described by London. However, the people were soon frightened by "the astonishing quickness with which this germ destroyed human beings, and [by] the fact that it inevitably killed any human body it entered. … From the moment of the first signs of it, a man would be dead in an hour. Some lasted for several hours. Many died within ten or fifteen minutes of the appearance of the first signs" (*1*). Through details of the course of the illness, London made the plague more realistic and even more frightening:

> "The heart began to beat faster and the heat of the body to increase. Then came the scarlet rash, spreading like wildfire over the face and body. Most persons never noticed the increase in heat and heartbeat, and the first they knew was when the scarlet rash came out. Usually, they had convulsions at the time of the appearance of the rash. But these convulsions did not last long and were not very severe. … The heels became numb first, then the legs, and hips, and when the numbness reached as high as his heart he died." (1)

London wrote of the rapid decomposition of corpses, which immediately released billions of germs, accelerating the spread of the disease and causing problems for the scientists who were not able to quickly find a specific treatment. By the time a serum against the plague was discovered, it was too late to stop the epidemic. Medicine and scientific progress were defeated by plague, as testified by the heroic death of bacteriologists who "were killed in their laboratories even as they studied the germ of the Scarlet Death. … As fast as they perished, others stepped forth and took their places" (*1*).

The defeat of the science and medicine in which the people had placed trust generated fear in the population. London gave detailed insight into the human reactions to the spread of the disease. In particular, Granser tells his grandsons how the people started to run away from the cities in a blind panic:

> "Thursday night the panic outrush for the country began. Imagine, my grandsons, people, thicker than the salmon-run you have seen on the Sacramento river, pouring out of the cities by millions, madly over the country, in vain attempt to escape the ubiquitous death. You see, they carried the germs with them. Even the airships of the rich, fleeing for mountain and desert fastnesses, carried the germs." (*1*)

Yet there was no escape. Germs were spreading, fast and uncontrolled. Nothing could stop it, and the world was in a state of sheer panic never experienced before. People started behave unreasonably: "we did not act in this way when ordinary diseases smote us. We were always calm over such things, and sent for the doctors and nurses who knew just what to do" (*1*). The population reacted to the outbreak of the plague in 2 ways: most tried in vain to isolate themselves and fled to avoid the contagion, whereas a minority, mainly rioters, begun drinking, robbing, and sometimes even killing:

> "In the midst of our civilization, down in our slums and labor-ghettos, we had bred a race of barbarians, of savages; and now, in the time of our calamity, they turned

> upon us like the wild beasts they were and destroyed us. And they destroyed themselves as well." (*1*)

After the plague, civilization fell apart, and the few survivors, scattered in a primitive world, had to fight for survival, echoing Darwinian theories: "Civilization was crumbling, and it was each for himself" (*1*). As had some earlier writers, London raised a harsh critique against the society that is seen as the ultimate cause of the world's destruction. In particular, in London's opinion, capitalism led to the rise in population and to overcrowding, and overcrowding led to plague. Consequently, capitalism is presented as the ultimate cause of the pandemic and thus harshly criticized.

As the human race in London's world was dying, the earth was being devastated by fires and conflagrations: "The smoke of the burning filled the heavens, so that the midday was as a gloomy twilight, and, in the shifts of wind, sometimes the sun shone through dimly, a dull red orb. Truly, my grandsons, it was like the last days of the end of the world" (*1*). The end of the world: this is how the pandemic was perceived. Not only did the people fear their own death but they also had the terrible feeling of being at the end of the world: the cities were being destroyed by fire; the people were fleeing away in hysteria. This immense panic grew even more, frightening and unprecedented because of the stop in communication with the rest of the world, a hopeless sign of death: "It was amazing, astounding, this loss of communication with the world. It was exactly as if the world had ceased, been blotted out" (*1*).

The brutality of the plague London presents is greater than that presented in previous works. The apocalyptic scenario illustrates a common fear of epidemics. In London's novel—as today—scientists were aware of the risk of uncontrolled pandemics. London's novel foresaw the first and most severe influenza pandemic in history, the Spanish influenza of 1918–1920, which began its spread only 6 years after the publication of The Scarlet Plague and caused the death of 20 million persons worldwide. In the novel, as in reality, human reactions to plague can vary greatly, but still all share a terrible fear, the fear of death—both as the end of one's life and as the end of civilization.

Conclusions

As London shows in his novel, pandemics can bring forth deeply rooted fears and modify human behavior greatly. The American novelist used the plague topos to criticize contemporary social structure: the destruction that follows the plague is both to be welcomed and despised. Indeed, the pandemic breaks the class barriers, but it also leads to the ruin of civilization. According to London's socialist values, only human brotherhood enables society to survive. Despite the political views of the author, the pandemic issue would have appealed to London's readers; in 1912, the American audience had recently experienced the San Francisco plague of 1900–1904, an epidemic of bubonic plague centered on San Francisco's Chinatown (*11*). During this epidemic, the initial denial and obstructionism of authorities in California, who wanted to prevent the loss of revenue from trade stopped by quarantine, were highly criticized by media and public opinion (*11*). Curiously, only 1 year before the publication of The Scarlet Plague, American writer and muckraker Samuel Hopkins Adams (1871–1958) wrote an editorial, Public Health and Public Hysteria, in the first volume of the Journal of the American Public Health Association (*12*). In his article, Adams argued that public health awareness is generated and sustained when fear of disease induces hysteria in population; consequently, at that time leprosy, cholera, and scarlet fever were considered the major public health priorities, rather than other, more common diseases, such as measles, whooping cough, and tuberculosis (*12*).

Today, despite the development of antimicrobial drugs, infectious diseases and germs continue to generate fear, as recently demonstrated by the worldwide epidemics of influenza A(H1N1) in 2009, avian influenza A(H5N1) in 2005–2006, and severe acute respiratory syndrome (SARS) in 2003, as well as the potential for attacks with bioterrorism agents such as anthrax or smallpox (*13*). Several studies have been conducted to analyze and hypothesize about the emotional, cognitive, and behavioral responses to epidemics among the public, in particular to provide policy makers and emergency responders with information about public perception and behavior in the aftermath of biological disasters, such as a deadly epidemic (*13,14*). A recent study in Switzerland analyzed the lay perceptions of collectives implicated in the 2009 influenza A(H1N1) outbreak and found that physicians and researchers were considered "heroes" of the pandemic (*15*). As in London's times, the study illustrated that the public placed trust mainly in scientists rather than in political authorities and states, which were thought to be partly ineffective (*15*). On the other hand, media and private corporations (e.g., the pharmaceutical industry), which are believed to take advantage of the spread of diseases and to create alarmism, are accused of being social "villains" (*15*), much as London criticized capitalists. However, recent outbreaks have demonstrated that even the scientific community may make mistakes in managing infectious disease (*16,17*), and during a pandemic, emotion and greed may affect not only the population but also scientific authorities and hospital workers. For example, as in the situation described by London, during the SARS epidemic, many heroic deeds were performed by scientists and health care workers, especially when SARS was an unknown microbiological enemy (*18,19*). Devotion to professional duty

resulted in a high level of camaraderie, cohesion, and encouragement in hospitals in Asia (*18*), as among the plague survivors in London's novel. However, the haunting fear of acquiring and spreading the disease to families, friends, and colleagues may also lead to understandable selfishness and cowardice in health providers (*20*). During the SARS crisis, for example, some physicians and nurses in Asia resigned, realizing that the profession was not for them (*18*).

Finally, London's work inspires reflection on the role of media during pandemics. In London's novel, newspapers, wires, and phone calls were the only tools for obtaining information on epidemic spread: "The man who sent this news, the wireless operator, was alone with his instrument on the top of a lofty building. […] He was a hero, that man who stayed by his post—an obscure newspaperman, most likely" (*1*). Today, the main sources of information on pandemics are widely available and include the mass media, such as television, radio, and print media such as magazines and newspapers; the Internet appears to be only partly used and mainly limited to younger age groups (*21*). In London's novel, the role of media seems to be positive (the "newspaperman" was looked upon as a hero as well as bacteriologists), but in modern times, the media are generally accused of exaggerating the risks of an epidemic and contributing to public misunderstandings of public health research evidence. Media reporting can sometimes appear to lower trust in scientific evidence, guiding public fear and spreading widely and almost instantaneously false information and exaggerated panic in public opinion (*22*). During the SARS outbreak, for example, propagation of redundant information and panic prompted reactions that were out of proportion to the risk posed by the disease (*23*). Media coverage can directly affect public risk perceptions, and recent studies have shown that media-triggered public concern may affect health-related personal measures taken during pandemics (*24*,*25*). International scientific literature has shown that, in more recent epidemics, media coverage may have had a positive influence on disease perception (*26*,*27*) and, in particular, on vaccination campaigns (*28*,*29*). As in London's novel, the media may be a useful resource in controlling epidemic fear, enabling a bridge to be created between government/science and public opinion (*30*).

Even though it was published a century ago, The Scarlet Plague presents the same concerns we face today, as demonstrated by the subsequent great success of this novel and the continuing literary topos of plague. Indeed, in the following decades, London's novel inspired other literary works, including Earth Abides by George R. Stewart in 1949, I Am Legend by Richard Matheson in 1954, and The Stand by Stephen King in 1978, as well as modern blockbuster movie such as 12 Monkeys (1995), 28 Days Later (2002), Carriers (2009), and Contagion (2011).

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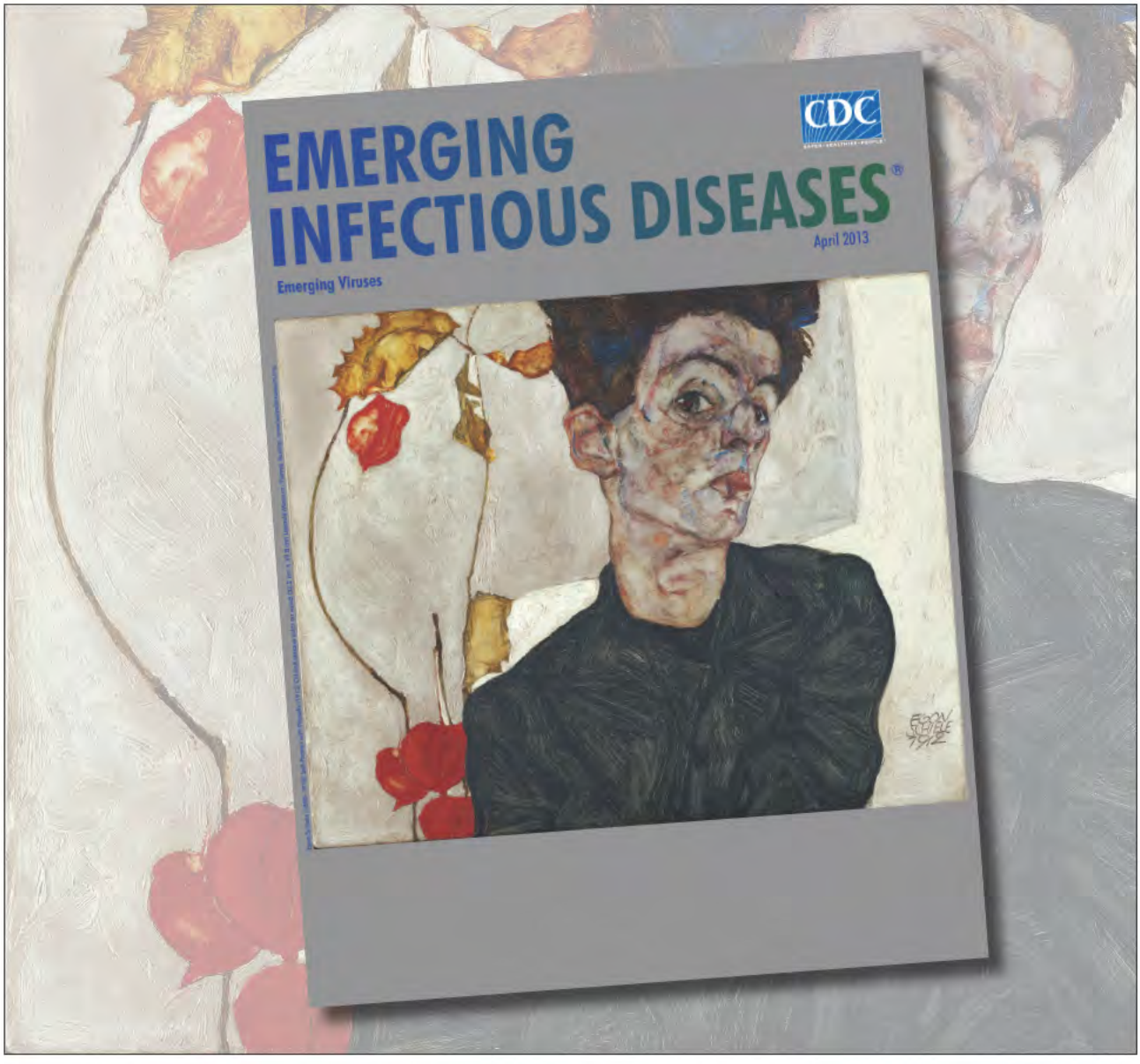

Identification of New Provisional Simian Adenovirus Species from Captive Monkeys, China

To the Editor: Adenoviruses commonly infect vertebrates, including humans and nonhuman primates (*1*). Adenoviruses from humans and animals were initially classified by differences in their agglutinating erythrocytes; now, however, they are often classified by sequence and phylogenetic analyses of their respective genes (*2*). One simian adenovirus species, SAdV-A, has been recognized by the International Committee on Taxonomy of Viruses (*2*). In addition, at least 25 simian adenovirus types have been identified (*3*). Since 2011, several novel adenoviruses have been isolated from nonhuman primates, including a group of new strains, designated SAdV-B, from asymptomatic rhesus macaques housed at 5 different primate facilities located across the United States (*2*,*4*).

Hexon is the most abundant protein in the icosahedral capsid, and the hexon gene is commonly analyzed to characterize and determine adenovirus types (*5*). We previously reported the detection of several new simian adenovirus strains in fecal specimens from captive monkeys in China (*3*). These novel strains were detected by using PCR and hexon gene–specific primers, but the product length (≈255 bp) may be insufficient to completely and accurately characterize these strains. Consequently, we further characterized 10 of these novel simian adenovirus strains by sequencing the entire open-reading frames of the hexon genes. Of the 10 strains, 9 were from rhesus macaque (CHN-8, CHN-14, CHN-23, CHN-30, CHN-36, CHN- 39, CHN-43, CHN-48, and CHN-51), and 1 was from a pigtail macaque (CHN-24).

We performed alignment analysis of the complete nucleotide hexon sequences of the 10 novel strains and of reference human and simian adenoviruses by using the MUSCLE program within the MEGA5 software package (*6*). To best fit the sequence data for the hexon gene, we used the DNA/Protein model test to identify the optimal evolutionary model, GTR+Γ+I (general time reversible + gamma + invariable). To construct the maximum-likelihood tree, we used the corrected Akaike Information Criterion and the MUSCLE program within MEGA5. Nucleotide and amino acid distance matrixes were prepared by using the *p*-distance algorithm of MEGA5.

Hexon genes of the 10 simian adenovirus strains ranged in length from 2,739 to 2,820 nt and from 913 to 940 aa. Four strains (CHN-8, CHN-39, CHN-43, and CHN-48) clustered with SAdV-A species, showing 81%–86% nt and 90%–93% aa identity (Figure). One strain (CHN-14) clustered with the newly identified SAdV-B species, showing 89% nt and 97% aa identity to strain SAdV-50. Two strains (CHN-30 and CHN-51) clustered with HAdV-G species, showing 87% nt and 93% aa and 85% nt and 94% aa identities, respectively, to strain HAdV-52. Three strains (CHN-23, CHN-24, and CHN-36) formed a new cluster that was not closely related to any of the previously reported strains. Comparison of the hexon gene sequences of simian adenovirus types identified in this study with human and simian adenovirus sequences from GenBank revealed 9 previously reported hypervariable regions (HVRs): HVR1–HVR9 (*5*,*7*). The simian adenovirus types within the same cluster were closely related in all 9 HVRs (data not shown).

For more accurate characterization and for possible species determination of adenovirus strains, it is beneficial to sequence the entire hexon gene. In our previous study (*3*), partial hexon sequence analyses showed that strains SAdV.Rh/CHN-23/2002 and SAdV.Rh/CHN-36/2002 (formerly named 23 M.m. and 36 M.m., respectively) clustered with HAdV-G species, and strain SAdV.pigtail/CHN-24/2002 (previously named 24 M.n.) clustered with SAdV-A species. However, sequencing of the complete hexon gene showed that the 3 strains appeared to be distinct from other strains and formed a potentially new species cluster. Thus, we propose a new candidate simian species, named SAdV-D, for this new cluster of macaque adenovirus strains.

Our phylogenetic analysis also identified another possible new simian species: 5 strains, which were previously reported as "unassigned adenoviruses," were isolated from Old World monkeys living in captivity and in the wild (*8*). We propose to designate this unassigned adenovirus cluster as candidate species SAdV-C (Figure). To definitively classify these species, further analyses of their entire genome sequences and organizations are needed. We also propose new strain designation nomenclature as follows: virus origin/isolation location plus strain identifier/isolation year. We believe that standard nomenclature and criteria are essential for identifying and classifying the increasing number of novel human and animal adenovirus strains that have been described on the basis of partial sequence data.

Our results demonstrated sequence conservation in the hexon genes of some adenovirus strains but also high diversity among other strains in a colony of captive monkeys. Our findings of close sequence relatedness between simian and human adenovirus strains suggest the interspecies transmission of these viruses and highlight the continuing risk for new and emerging infections in humans.

Kimberly R. Foytich, Garland Deshazer, Mathew D. Esona, Angela Liu, Yuhuan Wang, Xinming Tu, and Baoming Jiang

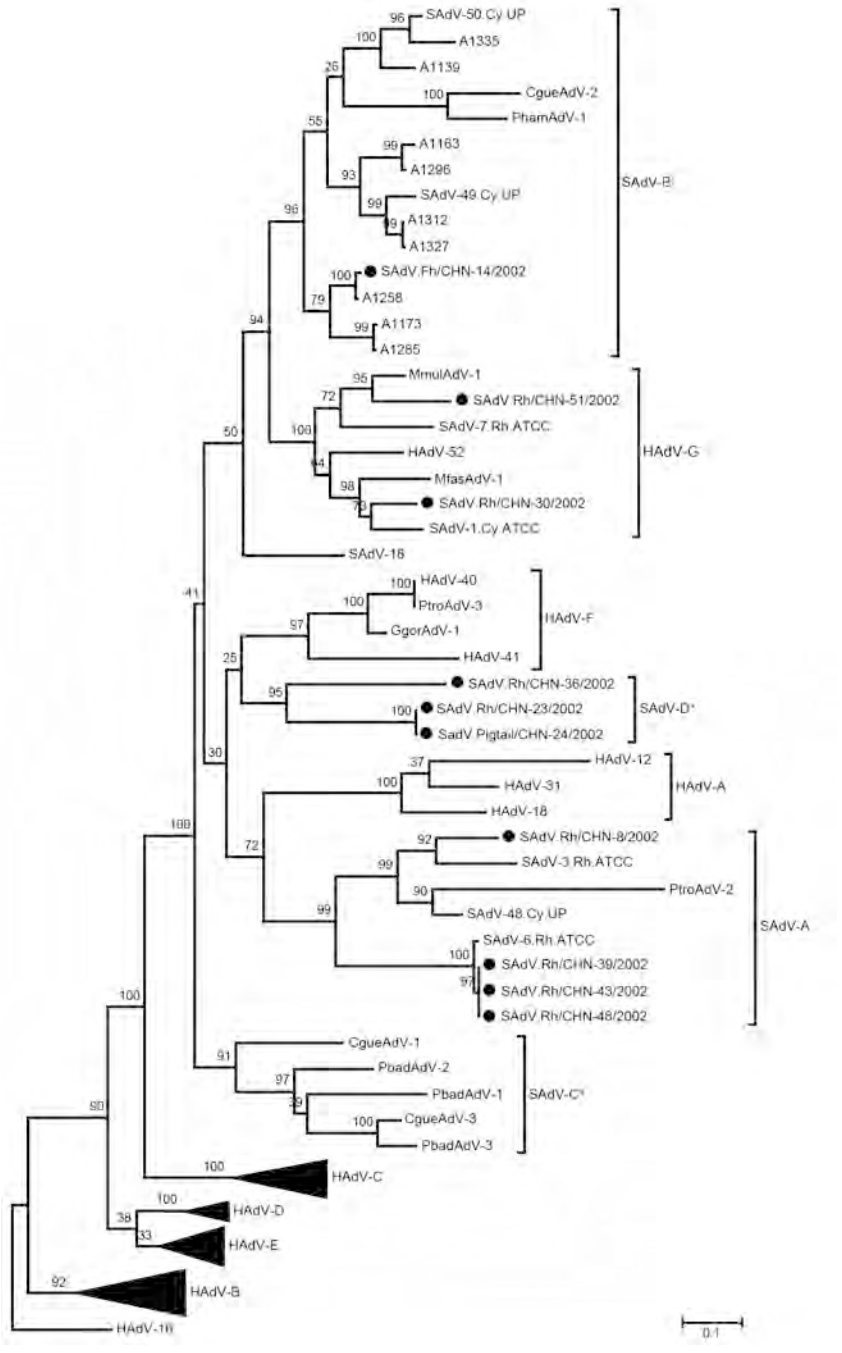

Figure. Phylogenetic analysis of complete hexon gene nucleotide sequences from human and simian adenoviruses. The maximum-likelihood tree was constructed as described in the text. Black dots indicate strains sequenced in this study; the sequences were deposited in GenBank (accession nos. KF053121–KF053130). Sequences for all reference strains used in the phylogenetic analysis were obtained from GenBank. Asterisks (*) indicate the proposed new candidate simian species. Numbers along branches and at nodes indicate bootstrap values. Scale bar indicates the branch lengths measured in the number of nucleotide and amino acid substitutions per site. AdV, adenovirus; ATCC, American Type Culture Collection; CHN, China; CgueAdV, *Colobus guereza* adenovirus; GgorAdV, *Gorilla gorilla gorilla* adenovirus; HAdV, human adenovirus; MfasAdV, *Macaca fascicularis* adenovirus; MmulAdV, *Macaca mulatta* adenovirus; PbadAdV, *Piliocolobus badius* adenovirus; Pham, *Papio hamadryas* adenovirus; PtroAdV, *Pan troglodytes schweinfurthii* adenovirus; and SAdV, simian adenovirus.

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Three-Toed Sloth as Putative Reservoir of *Coxiella burnetii*, Cayenne, French Guiana

To the Editor: Q fever is an emerging zoonosis and a major public health concern in French Guiana, a French overseas region located on the northeastern coast of South America (*1*,*2*). Most cases occur in the city of Cayenne (*3*), specifically in the suburbs, where houses are near wooded hills (*4*). Genotyping performed by using multispacer sequence typing showed that MST17, a unique genotype of *C. burnetii*, circulates in Cayenne and is responsible for epidemics of Q fever (*5*). *C. burnetii* transmission peaks during the rainy season, and the incidence of Q fever usually increases 1–3 months later (*6*). The animal reservoir of *C. burnetii* in French Guiana is unknown; previous studies have excluded domestic ruminants, which are known to be *C. burnetii* reservoirs elsewhere in the world (*6*). Four serologic surveys showed few *C. burnetii*–positive opossums, dogs, rodents (*Proechimys* spp.), bovines, or birds in French Guiana (*7*). In 2013, using real-time PCR (qPCR) analysis of vaginal swab samples, we showed that 6/158 (3.8%) dogs from Cayenne and 0/206 bats from the coastal area of French Guiana were positive for *C. burnetii* (Cycle threshhold [C_t]<35). One of the positive samples was identified as genotype MST17 (*5*). A case–control study among humans identified several risk factors for Q fever, including living near a forest and the presence of wild animals near the house (*6*).

During January–April 2013, a Q fever outbreak occurred in Tiger Camp, a military residential area located at the top of a wooded hill in Cayenne. Vaginal swab samples were collected from animals living in the area (13 goats, 8 sheep, 7 bats, 34 birds, 2 opossums, 4 iguanas, and 17 geckos); all samples were negative for *C. burnetii* by qPCR. In addition, serologic tests for *C. burnetii* were negative for samples from all 37 small ruminants maintained near the outbreak area.

In January 2014, a dead (accidental death) female 3-toed sloth (*Bradypus tridactylus*) (Figure, panel A) was found on the road near the residence of a Q fever patient. We retrieved the sloth and collected feces, spleen, liver, kidney, lung, and uterus samples and a vaginal swab sample. A total of 16 ticks were removed from the sloth and stored in 70% alcohol.

DNA was extracted from the feces, organs, and ticks by using the BioRobot EZ1 Workstation (QIAGEN, Courtaboeuf, France). qPCR targeting the repeated insertion sequence IS*1111* was performed by using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Marne la Coquette, France) as described (*8*). We confirmed all positive results by performing a second qPCR targeting the IS*30a* repeated sequence. DNA samples with C_t values ≤35 in both assays were considered positive for *C. burnetii*. A standard calibration curve quantifying the target IS*1111* was generated by using 10-fold serial dilutions of *C. burnetii* Nine Mile strain. The number of IS*1111* intergenic sequences found in the genome of strain *C. burnetii* MST17 was identical to that for the Nine Mile strain (F. D'Amato, unpub. data); thus, the qPCR that we used was valid for quantifying the number of *C. burnetii* MST17 IS1111 copies/mL in samples we collected (*5*).

qPCR analysis showed that the feces were highly positive for *C. burnetii*; the sample had a low C_t value of 23, corresponding to 7 $\log_{10}$ DNA copies/mL (*9*). The spleen was also positive for *C. burnetii*; the C_t value was 34, corresponding to 3.6 $\log_{10}$ DNA copies/mL. Results for the other samples were negative.

Using morphologic criteria, we identified all 16 ticks collected from the sloth as *Amblyomma geayi* (Figure,

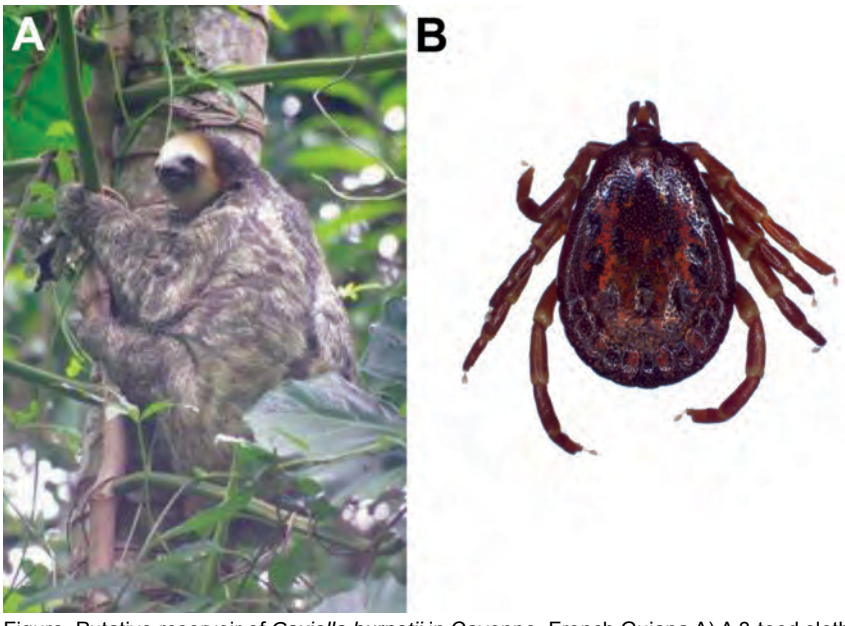

Figure. Putative reservoir of *Coxiella burnetii* in Cayenne, French Guiana A) A 3-toed sloth (*Bradypus tridactylus*) in its natural habitat in Tiger Camp, a military residential area located at the top of a wooded hill in Cayenne, French Guiana (photograph by S. Fernandes). B) A male tick (*Amblyomma geayi*) found on the 3-toed sloth in this study (photograph by J.M. Berenger). A color version of this figure is available online (http://wwwnc.cdc.gov/eid/article/20/10/14-0694-F1.htm).

panel B). We performed *C. burnetii*–specific qPCR on the ticks; 14 (88%) were positive.

We genotyped *C. burnetii*–positive DNA from the feces and from 6 of the 16 ticks by using multispacer sequence typing as described (*5*). All samples were identified as MST17, the unique genotype circulating in Cayenne (*5*).

After obtaining the laboratory results, we confirmed that a local group in charge of the collection and treatment of injured animals usually released rehabilitated 3-toed sloths into Tiger Camp. Residents of Tiger Camp regularly observed and came into contact with the sloths, and ticks were frequently observed on the fur of the animals. Furthermore, 3 Q fever patients from Cayenne reported contact with sloths.

Feces from the sloth in this study were highly infectious for *C. burnetii*. Because sloths live in tall trees and can shed this bacterium in their feces, human contamination might occur through inhalation of infectious aerosols from feces. The high prevalence of *C. burnetii* infection in ticks also suggests possible transmission through tick bites or from aerosols of tick feces that have been deposited on the skin of animal hosts; such feces can be extremely rich in bacteria and highly infectious (*10*).

In this 2013 outbreak of Q fever, epidemiologic studies led to the identification of 3-toed sloths as a putative source of *C. burnetii* infection. Further investigations are needed to confirm the role of sloths as a reservoir for *C. burnetii* in French Guiana and to implement efficient measures to prevent transmission to humans.

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Marburgvirus Resurgence in Kitaka Mine Bat Population after Extermination Attempts, Uganda

To the Editor: Marburg virus (MARV) and Ravn virus (RAVV), collectively called marburgviruses, cause Marburg hemorrhagic fever (MHF) in humans. In July 2007, 4 cases of MHF (1 fatal) occurred in miners at Kitaka Mine in southern Uganda. Later, MHF occurred in 2 tourists who visited Python Cave, ≈50 km from Kitaka Mine. One of the tourists was from the United States (December

2007) and 1 was from the Netherlands (July 2008); 1 case was fatal (*1*,*2*,*3*). The cave and the mine each contained 40,000–100,000 *Rousettus aegyptiacus* bats (Egyptian fruit bats).

Longitudinal investigations of the outbreaks at both locations were initiated by the Viral Special Pathogens Branch of the Centers for Disease Control and Prevention (CDC, Atlanta, GA, USA, and Entebbe, Uganda) in collaboration with the Uganda Wildlife Authority (UWA) and the Uganda Virus Research Institute (UVRI). During these studies, genetically diverse MARVs and RAVVs were isolated directly from bat tissues, and infection levels of the 2 viruses were found to increase in juvenile bats on a predictable bi-annual basis (*4*,*5*). However, investigations at Kitaka Mine were stopped when the miners exterminated the bat colony by restricting egress from the cave with papyrus reed barriers and then entangling the bats in fishing nets draped over the exits. The trapping continued for weeks, and the entrances were then sealed with sticks and plastic. These depopulation efforts were documented by researchers from UVRI, the CDC, the National Institute of Communicable Diseases (Sandringham, South Africa), and UWA during site visits to Kitaka Mine (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/8/14-0696-Techapp1.pdf). In August 2008, thousands of dead bats were found piled in the forest, and by November 2008, there was no evidence of bats living in the mine; whether 100% extermination was achieved is unknown. CDC, UVRI, and UWA recommended against extermination, believing that any results would be temporary and that such efforts could exacerbate the problem if bat exclusion methods were not complete and permanent (*6*,*7*).

In October 2012, the most recent known marburgvirus outbreak was detected in Ibanda, a town in southwest Uganda. Ibanda is ≈20 km from the Kitaka Mine and is the urban center that serves smaller communities in the Kitaka area. This MHF outbreak was the largest in Ugandan history: 15 laboratory-confirmed cases occurred (*8*). In November 2012, an ecologic investigation of the greater Ibanda/Kitaka area was initiated. The investigation included interviews with local authorities to locate all known *R. aegyptiacus* colonies in the area. Although minor colonies of small insectivorous bats were found, the only identifiable colony of *R. aegyptiacus* bats was found inside the re-opened Kitaka Mine, albeit at much reduced size, perhaps 1%–5% of that found before depopulation efforts.

To determine whether the *R. aegyptiacus* bats that had repopulated Kitaka Mine were actively infected with marburgviruses, we tested 400 bats by using previously described methods (*4*,*5*). Viral RNA was extracted from ≈100 mg of liver and spleen tissue by using the MagMAX Total Nucleic Acid Isolation Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's recommended protocol. The Fisher exact test was conducted by using IBM SPSS Statistics, version 19.0 (IBM Corp., Armonk, NY, USA).

Of the 400 *R. aegyptiacus* bats collected, 53 (13.3%) were positive for marburgvirus RNA by quantitative reverse transcription PCR (32/233 [13.7%] adults and 21/167 [12.6%] juveniles; online Technical Appendix Table); marburgvirus was isolated from tissue samples from 9 of the 400 bats. The overall level of active infection was significantly higher than that found in Kitaka Mine during 2007–2008 (5.1%) (*5*) (Fisher exact test, $p<0.001$) and in other studies in Uganda (Python Cave [2.5%]) and Gabon (4.8%) (*4*,*9*). The reason for the increase is not clear, but it may be related to the effects of the extermination and subsequent repopulation. Increases in disease prevalence in wildlife populations after culling are not unprecedented (*6*,*7*). We speculate that after the depopulation attempt, a pool of susceptible bats became established over time and was subjected to multiple marburgvirus introductions, as evidenced by the genetic diversity of viruses isolated from the bats (Figure). A pool of susceptible bats would have led to higher levels of active infection within the colony, thereby increasing the potential for virus spillover into the human population. A significant sex and age bias was not detected with respect to active infection during the breeding season (Fisher exact test, $p>0.5$ for both), and overall, the presence of virus-specific IgG among the bats was 16.5%, a finding consistent with that in previous studies (*4*,*5*).

Phylogenetic analysis of viral RNA genome fragment sequences in this study showed high marburgvirus genetic diversity, including the presence of RAVVs and MARVs. Sequences for isolates from 3 bats were nearly identical to those of the MARV isolates obtained from patients in the 2012 Ibanda outbreak (*8*), suggesting that bats from Kitaka Mine were a likely source of the virus.

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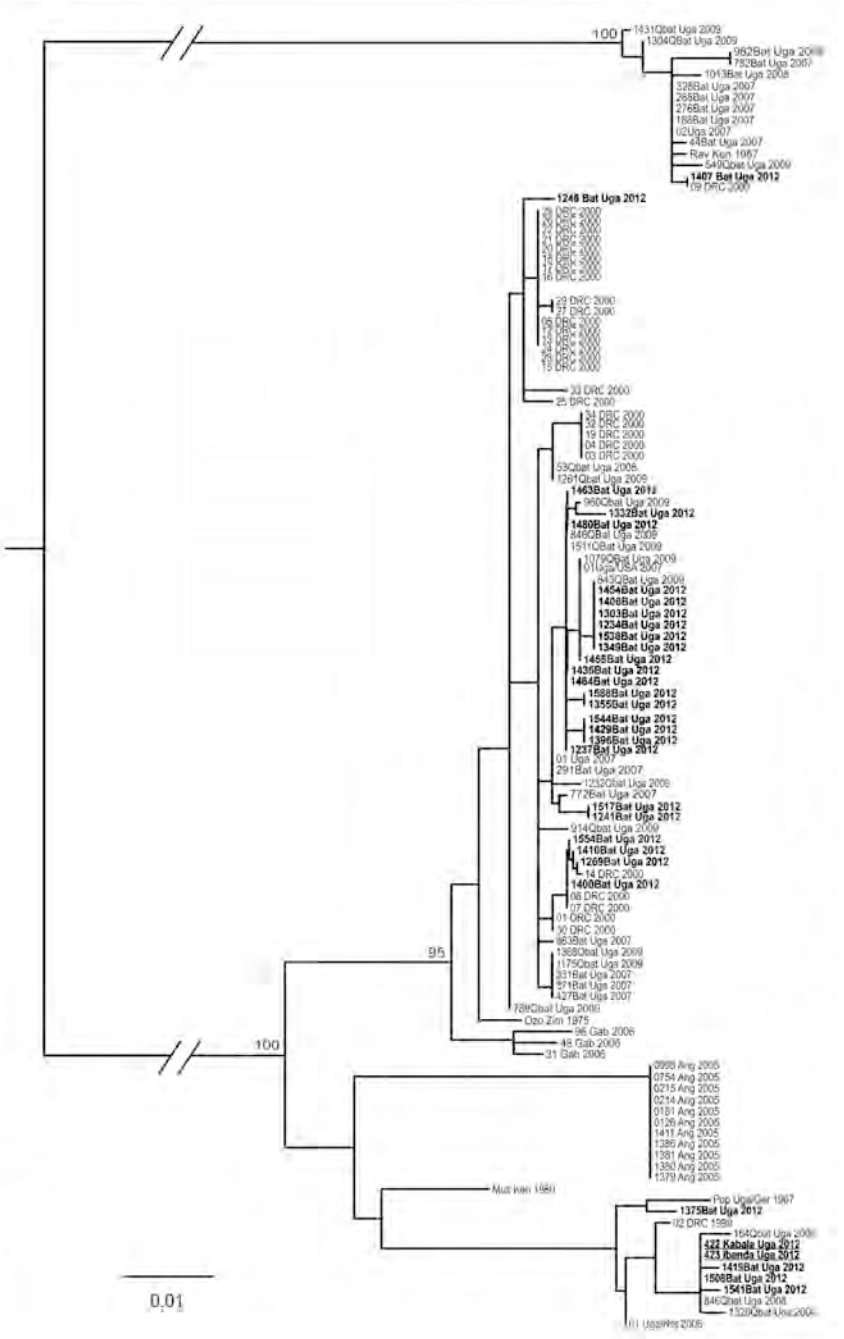

Figure. Phylogeny of concatenated marburgvirus nucleoprotein (NP) and viral protein 35 (VP35) gene fragments as determined by using the maximum-likelihood method. Sequences from the NP (289–372 nt) and VP35 (203–213 nt) genes were amplified and determined from viral RNA and then sequenced as described elsewhere (*4*). Sequence names in boldface represent those generated from samples collected from bats during the November 2012 outbreak investigation at Kitaka Mine, Uganda. Underlined sequence names represent those generated from samples obtained from marburgvirus-infected persons in Kabale and Ibanda, Uganda, in 2012. Multiple sequence alignments were generated, and a maximum-likelihood analysis was conducted on concatenated NP and VP35 (208–580 nt) sequences by using the PhyML method in conjunction with the GTR+I+G nucleotide substitution model implemented in SeaView version 4.2.12 (*10*). NP and VP35 gene sequences determined from samples in this study (in boldface) were submitted to GenBank (accession nos. KJ747211–KJ747234 and KJ747235–KJ747253, respectively). Bayesian posterior probabilities above 50 are shown at the nodes. Scale bar indicates nucleotide substitutions per site. Ang, Angola; DRC, Democratic Republic of Congo; Gab, Gabon; Ger, Germany; Ken, Kenya; Net, Netherlands; Rav, Ravn virus; Uga, Uganda; Zim, Zimbabwe.

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Detection of Measles Virus Genotype B3, India

To the Editor: Molecular epidemiologic investigations and virologic surveillance contribute notably to the control and prevention of measles (*1*). Nearly half of measles-related deaths worldwide occur in India, yet virologic surveillance data are incomplete for many regions of the country (*2*,*3*). Previous studies have documented the presence of measles virus genotypes D4, D7, and D8 in India, and genotypes D5, D9, D11, H1, and G3 have been detected in neighboring countries (*3*,*4*).

Kerala, India's southernmost state, has high measles vaccination coverage compared with many other states in the country; however, the disease is still endemic in the region. Two districts, Thiruvananthapuram and Malappuram, report the highest numbers of cases (*5*). Baseline data on circulating measles virus genotypes are needed for measles elimination, but such data are not available for Kerala. In this context, we performed a pilot genetic analysis of the measles virus strains circulating in Thiruvananthapuram, the capital of Kerala. We used throat and nasopharyngeal swab and serum samples from children admitted to Sree Avittom Thirunal Hospital during measles outbreaks occurring March–August 2012.

We used the Vero/human-SLAM cell line (http://www.phe-culturecollections.org.uk). for isolation of measles virus from throat and nasopharyngeal swab samples. For serologic confirmation of cases, we used a commercial measles IgM ELISA kit (IBL International GmbH, Hamburg, Germany). Virus genotyping was based on the 450-nt coding sequence for the carboxyl terminus of nucleoprotein (N) of measles virus, as recommended by the World Health Organization (*3*,*6*). We extracted RNA from the samples using TRIzol reagent (GIBCO-BRL, Grand Island, NY, USA). We performed reverse transcription PCR using a SuperScript One-Step RT-PCR kit with a Platinum *Taq* system (Invitrogen, Carlsbad, CA, USA) and previously described primers (*3*,*6*). Amplicons were subjected to bidirectional sequencing using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). We edited and aligned nucleotide sequences using Bio Edit 7.1.11 software (*7*). Phylogenetic analysis was performed by using the maximum-likelihood method implemented in the MEGA5 program (*8*) to compare the determined N gene sequences with the World Health Organization reference sequences of the 24 known measles genotypes.

PCR products could be amplified from 16 of the 24 samples analyzed. Ten samples provided high quality sequence reads for the N gene coding region, which were used for further analysis. Clinical and demographic data for these 10 cases, virus isolation status, and GenBank accession numbers of the sequences are summarized in the Table.

Phylogenetic analysis revealed 1 of the 10 measles virus strains to be of genotype D8 (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/10/13-0742-Techapp1.pdf), a genotype previously found to be circulating in Kerala and in other regions of India (*3*,*6*,*9*,*10*). The other 9 virus strains were closely related to B3 genotype reference strains, indicating circulation of the B3 genotype in Kerala (online Technical Appendix Figure 1). The nucleotide sequences of 7 of the 9 strains were identical, indicating a single chain of transmission. The remaining 2 samples showed sequence divergence, indicating independent sources of infection. In a phylogenetic analysis comparing the Kerala B3 genotypes and a dataset of

Table. Details of the clinical samples used for genotype analysis of measles virus strains, Kerala, India, 2012

Strain name*	Clinical details of patients					GenBank accession no.
	Age	Sex	Measles vaccination status	Contact history	Complications	
MVi/Thiruvananthapuram.IND/12.12[B3]	11 mo	F	No	Yes	Pneumonia	KC997602
MVs/Thiruvananthapuram.IND/14.12/1[B3]	9 mo	M	No	Yes	Pneumonia	KC997603
MVs/Thiruvananthapuram.IND/14.12/2[B3]	3 y	M	Yes	Yes	None	KC997604
MVi/Thiruvananthapuram.IND/14.12/3[B3]	6 y	M	No	No	None	KC997605
MVs/Thiruvananthapuram.IND/14.12/4[B3]	7 mo	M	No	Yes	Pneumonia	KC997606
MVi/Thiruvananthapuram.IND/15.12[B3]	10 mo	M	Yes	Yes	None	KC997607
MVs/Thiruvananthapuram.IND/30.12[B3]	7 mo	F	No	No	Pneumonia	KC997608
MVs/Thiruvananthapuram.IND/31.12/1[B3]	9 mo	F	No	Yes	Pneumonia	KC997609
MVs/Thiruvananthapuram.IND/31.12/2[B3]	2 y	F	No	Yes	Pneumonia	KC997610
MVs/Thiruvananthapuram.IND/34.12[D8]	10 y	M	No	No	None	KC997611

*Named according to World Health Organization nomenclature following the scheme: measles virus (MV), isolate (i) or clinical specimen (s)/place. country/epidemiologic week.year/sample identity [genotype].

global measles B3 genotypes selected from GenBank, the strains from Kerala formed a separate cluster (online Technical Appendix Figure 2). This cluster also contains a strain from Germany (MVs/Regensburg.DEU/37.12/). The strains in this cluster show close identity to a measles strain identified in the state of New York, USA. A search of the MeaNS (Measles Nucleotide Surveillance) database revealed that the Kerala B3 sequences had the closest match to the strain isolated in Germany mentioned above and also to a strain from the Sultanate of Oman (MVs/Muscat.OMN/38.11/).

The B3 genotype identified in this study could be a previously undetected genotype endemic to India or a recent importation. B3 is endemic to many countries in Africa, and its importation into Europe and North America and elsewhere has been described (*4*). Further studies with samples from a wider geographic area of Kerala are required to determine the spread and genetic diversity of these strains and ascertain their relationship to the global B3 strains. It would also be of interest to determine whether B3 strains co-circulate with D8 strains, whether they will eventually replace D8 as the predominant genotype in Kerala, or if they will cease to exist as the outbreak diminishes. This report underscores the need for systematic nationwide measles virus surveillance in India to identify all endemic virus genotypes and to monitor importation of new virus strains from other countries.

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O'nyong-nyong Virus Infection Imported to Europe from Kenya by a Traveler

To the Editor: O'nyong-nyong virus (ONNV) is a mosquitoborne RNA virus of the *Togaviridae* family. The virus was first isolated in June 1959 from serum samples from febrile patients in the northern province of Uganda (*1*). Unlike other members of the alphavirus genus, ONNV is primarily transmitted by anopheline mosquitoes (*2*). ONNV is genetically and serologically related to chikungunya virus (CHIKV) (*1*), but is restricted to the African continent. The clinical picture resembles CHIKV infection, i.e., a self-limited febrile illness characterized by headache, rash, and joint pain. In contrast to CHIKV, ONNV is reported to cause lymphadenopathy more often and affected joints do not show effusions (*3*).

ONNV caused 2 large-scale epidemics in East Africa during 1959–1962 and in 1996. The first instance had spread from Uganda south to Mozambique and westward to Senegal. Comprising >2 million cases in east Africa alone, this first epidemic ranked among the largest mosquitoborne virus outbreaks recorded (*4*). After an absence of reported cases for 35 years, a second ONNV epidemic occurred in Uganda (*3*–*4*). Patients had fever, a maculopapular rash, pruritis, myalgia, and arthralgia of large joints. Lymphadenitis, most often of the posterior cervical spine region, was also observed (*3*). Despite the virus' potential to cause large outbreaks and its endemicity in the vast geographic area of East Africa, and at least sporadic occurrence in West Africa, imported cases to other areas have not been reported.

On October 14, 2013, a 60-year-old woman residing in Germany who had returned home 2 days before from a 7-week vacation in East Africa sought medical attention at the University Medical Center, Section of Clinical Tropical Medicine, in Heidelberg for recurring fever and illness that began during her travel. She and her husband had traveled from Kenya to Uganda, Rwanda, Tanzania, and back to Kenya, along the shore of Lake Victoria. Bed nets and malaria prophylaxis were used regularly. On October 9, she had experienced the first episode of fever, general malaise, arthralgia, and nausea while staying at the lake shore near the city of Kisumu, Kenya. Fever had persisted until October 12. Thin and thick blood films, examined in a local hospital and later in Nairobi, did not show malarial parasites.

October 14 was day 5 of symptom onset. Her fever reached 39°C and lasted 3 more days. It was accompanied by cervical spine and nuchal lymphadenopathy, nausea, and arthralgia of the small joints of her hands and feet. A maculopapular rash developed, which covered her face, hands, feet, and trunk. Her face, hands, and feet were edematous. Laboratory tests on admission to the medical center revealed a slightly elevated C-reactive protein level of 13 mg/L (reference level<5). Full blood count and results of liver function tests were within reference ranges. Thin and thick blood films were examined again and were negative for *Plasmodium* spp. A serum sample from the day of admission showed anti-ONNV IgM and IgG and anti-CHIKV IgM and IgG in the indirect immunofluorescence assay, according to Tappe et al. (*5*, Table). Serology for dengue virus and generic alphavirus reverse transcription PCR (*6*) were negative. A 4-fold anti-ONNV IgG titer decrease in the indirect immunofluorescence assay was demonstrated in the second serum sample, which was collected 26 days after disease onset (Table). The presence of ONNV-specific neutralizing antibodies in the second serum sample was confirmed by a virus neutralization test. Cross-neutralizing antibodies against CHIKV were detected also, but with a notably lower titer (1:80) when compared with the ONNV titer (1:1,280) (Table). Ten days after symptom onset, the patient recovered spontaneously. Her husband had no symptoms of illness during travel or after returning.

We report the laboratory-confirmed case of an ONNV infection imported into Europe. This patient most likely was infected in the eastern part of Kenya (Kisumu region), where she had stayed during the 2 weeks before symptom onset. The case highlights the fact that ONNV infections, which occur sympatrically with CHIKV infections in East Africa, lead to symptoms resembling CHIKV infection. The clinical and laboratory findings emphasize the importance of a careful diagnostic and clinical assessment of travelers

Table. Results of serologic analysis of a German traveler from Kenya with O'nyong-nyong virus infection, October 2013*

	Immunofluorescence assay†				
	5 d after symptom onset		26 d after symptom onset		Virus neutralization test,
Virus	IgG	IgM	IgG	IgM	26 d after symptom onset
O'nyong-nyong	1:160	1:160	1:2,560	1:1,280	1:1,280
Chikungunya	1:80	1:80	1:2,560	1:320	1:80
Sindbis	Neg	Neg	1:640	Neg	ND
Semliki Forest	Neg	Neg	Neg	Neg	ND

*Neg, negative; ND, not done.
†Immunofluorescence assay and virus neutralization test results of <1:20 were considered negative.

with suspected arboviral disease, and consideration of the well-known serologic cross-reactions in the alphavirus group. Because of the serologic and clinical similarities of ONNV and CHIKV infections, it remains unclear how many true ONNV infections in travelers have been diagnosed as CHIKV infections. Similar to other arboviruses, especially CHIKV and dengue viruses (*7*,*8*), ONNV might have the potential to spread to areas outside of Africa. There are no known invasive anopheline vectors for ONNV in Europe, but it was demonstrated that the culicine mosquito species *Aedes aegypti*, found in some parts of Europe (*8*), might be a competent vector for ONNV (*9*). Thus, it will be critical to study the vector competence of both the indigenous anopheline and culicine mosquitoes for ONNV in Europe.

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This letter is dedicated to the late Ursula Herrmann (1927–2014) for making this study possible.

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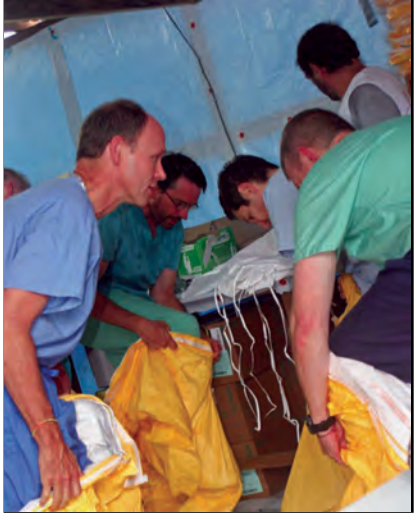

Rickettsia felis and Changing Paradigms about Pathogenic Rickettsiae

To the Editor: Mediannikov et al. recently reported several features common to the epidemiology of *Rickettsia felis* infection and malaria in Africa (*1*). Similar to the findings of several other recent studies in Africa (*2*,*3*), the authors diagnosed *R. felis* infection in febrile—and to a lesser extent in afebrile—persons by detecting *R. felis* DNA in human blood samples processed by highly sensitive real-time PCR. These results challenge some paradigms in rickettsiology that need to be more critically evaluated.

Because *R. felis* DNA was detected in circulating blood of asymptomatic persons (albeit more frequently in patients with mild febrile illness), Mediannikov et al. proposed that humans could be a natural reservoir of *R. felis*, as they are for malaria parasites. *R. felis* antibodies failed to develop in nearly all patients in whom *R. felis* DNA was detected, even after repeated detection of *R. felis* DNA. In 2 other studies, the same researchers proposed that patients might have several episodes of *R. felis* infection (relapse or reinfection) to explain why DNA of the agent was detected in the blood at multiple times (*2*,*3*). They also proposed that the absence of an antibody response would explain why the disease relapses in some persons (*3*).

These changing paradigms in rickettsiology require thorough evaluation. Once inside a vertebrate host, pathogenic rickettsiae have been believed to multiply primarily within endothelial cells in the patient's organs. As far as we know, rickettsiae do not multiply within circulating blood cells (*4*). In contrast, the agents of malaria (*Plasmodium* spp.) are typically parasites of erythrocytes. Therefore, a blood sample from a person with malaria is an excellent source for PCR diagnostic testing. The sensitivity of PCR for rickettsiae in human blood samples is very low because the sensitivity depends on the magnitude of the vasculitic lesions, i.e., the number of endothelial cells destroyed or detached by rickettsial growth, resulting in circulating rickettsiae. *R. conorii* (*5*) and *R. rickettsii* (*6*) were detected by highly sensitive PCR in 100% of fatal cases and in only very few nonfatal cases.

In addition to never having been isolated from humans, *R. felis* has many characteristics of a symbiotic organism. It possesses a mosaic structure genome (size 1.48 Mb) with a high coding capacity (83%) that is typical of symbiotic bacteria (*7*). Merhej et al. have proposed that within a given bacterial genus (including *Rickettsia*), pathogenic species have smaller genomes than nonpathogenic species (*8*). In the genus *Rickettsia*, the pathogens *R. rickettsii*, *R. prowazekii*, *R. sibirica*, *R. typhi*, *R. parkeri*, and *R. conorii* have genomes of ≈1.2–1.3 Mb, whereas the apparently nonpathogenic *R. bellii* has a 1.5-Mb genome, similar to that of *R. felis*. In contrast to the well-known pathogenic *Rickettsia* species, *R. felis* has been reported in a variety of invertebrate hosts, including hematophagous (fleas, ticks, flies, mosquitoes) and non-hematophagous (book lice) arthropods (*9*). Behar et al. have suggested that *R. felis* is responsible for inducing parthenogenesis in book lice, similar to the manner of *Wolbachia* organisms in various invertebrate hosts (*9*). Furthermore, *R. felis* forms mycetomes in book lice, a growth feature typical of bacterial endosymbionts (*10*).

The current view in rickettsiology has a strong anthropocentric bias because the studies have concentrated on parasitic arthropods that feed on humans rather than on free-living arthropods. In fact, the number of *Rickettsia* species associated with non-hematophagous hosts might be much greater than the ones of medical importance (*9*). Thus, considering *R. felis* as an important pathogen in Africa (and in the world) might be premature. Several questions need to be answered before such a conclusion. In asymptomatic persons in whom endothelial cells are likely to be intact, where does *R. felis* grow to be released at detectable levels in the circulating blood? Considering that all classical spotted fever agents induce an antibody response (*4*), why do *R. felis* antibodies fail to develop in humans after a clinical illness attributed to *R. felis*? In addition, repeated reports that the main vector of *R. felis* is the cat flea, *Ctenocephalides felis*, need to be proven by experimental demonstration of its vector capacity.

Given the numerous questions about *R. felis*, we would add another: could *R. felis* be a symbiont of a human parasite, such as a protozoon or a helminth? Obviously, the answer is unknown. However, had we not known that *Wolbachia* organisms are typically endosymbiotic bacteria of both human and animal filarial nematodes, what would we conclude if we detected *Wolbachia* DNA in blood of either asymptomatic or ill patients?

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Pulmonary Disease Caused by *Mycobacterium marseillense*, Italy

To the Editor: *Mycobacterium marseillense* was recently described as a new species belonging to the *Mycobacterium avium* complex (MAC) (*1*). We describe a case of pulmonary disease caused by *M. marseillense* in an immunocompetent patient. All strains isolated from the patient were preliminarily identified as *M. intracellulare*; however, a retrospective molecular analysis corrected the identification to *M. marseillense*.

In December 2005, a 65-year-old man was admitted to the University Hospital, Modena, Italy, with a 2-week history of fever, cough, and hemoptysis. Physical examination detected diffuse rales, and chest radiographs showed a diffuse nodular opacity and bronchial thickening, confirmed by high-resolution computed tomography (CT) of the chest (Figure, panel A). The patient had experienced several previous episodes of hemoptysis and persistent productive cough since 1998, and tubular bronchiectasis had been detected on previous high-resolution CT images. The patient had a history of thalassemia minor, was HIV negative, and was formerly a mild smoker (10 cigarettes/day for 4 years during his youth). He had no chronic disorders and no history of immunosuppressive-drug or alcohol use.

Bacterial and fungal cultures and a smear for acid-fast bacilli performed on a bronchoalveolar lavage (BAL) sample were all negative. A nontuberculous mycobacterium strain was isolated by culture and preliminarily identified as *M. intracellulare* by using the GenoType Mycobacterium CM/AS Kit (Hain Lifesciences, Nehren, Germany). At that time, a drug susceptibility test for isoniazid, rifampin, streptomycin, and ethambutol was improperly performed (i.e., was not applicable for MAC) by using the agar proportion method; sensitivity information for macrolides was unavailable. The strain was resistant to ethambutol and susceptible to the other drugs. The physician prescribed rifampin, isoniazid, and amikacin. After remission of fever and hemoptysis and improvement of chronic cough, the patient was discharged from the hospital.

In March 2006, he was readmitted to the hospital for worsening of his condition and onset of side effects associated with rifampin and isoniazid use. The treatment was discontinued and replaced by levofloxacin, terizidone, and azithromycin, which resulted in remission of symptoms. This therapy was continued after hospital discharge.

In 2007, the patient was twice admitted for follow-up and microbiological testing to determine bacteriologic status. All 3 separate sputum samples were negative for mycobacteria, other bacteria, and fungi. However, BAL sample culture results were positive for the same mycobacterium despite continued therapy with levofloxacin, terizidone, and azithromycin.

During 2008, as an investigation of the possibility of persistent excretion of organisms, additional samples were collected 5 times. The sputum cultures were intermittently positive, while the BAL sample cultures were persistently positive.

In May 2009, after the patient had been persistently stable and had negative culture results for 14 months, the antimicrobial drug therapy was stopped. In December 2010, the patient's only symptom was persistent productive cough; however, the sputum culture was again positive, and high-resolution CT revealed a worsening condition of his lungs (Figure, panel B). A new antimycobacterial drug regimen of ethambutol, rifampin, and azithromycin was started, in accordance with the international

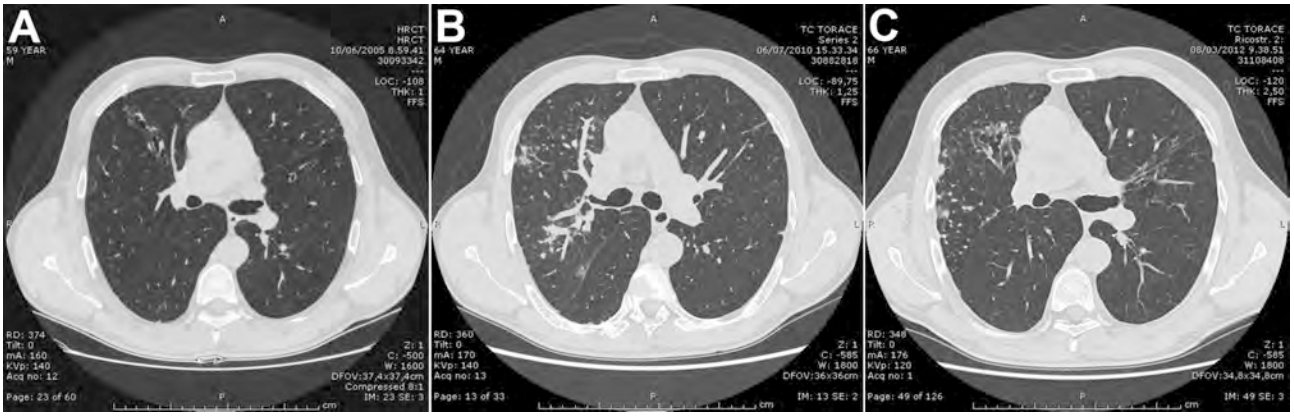

Figure. High-resolution computed tomographic chest images of a man with prolonged pulmonary disease caused by *Mycobacterium marseillense,* Italy. A) October 6, 2005. Bilateral bronchiectasis, mainly in the middle lobe and lingula, associated with multiple nodules and middle lobe consolidation. B) June 7, 2010. Increased micronodular opacities, mainly in the right middle and lower lobes, and a worsening of the bronchiectasis. C) August 3, 2013. Persistence of nodular component, cavitation, and wider bronchiectasis with bronchial wall thickening.

guidelines of the American Thoracic Society and the Infectious Diseases Society of America (*2*).

After the patient had received 6 months of therapy, the sputum culture result was again negative. The acid-fast bacilli smear and culture results remained negative until November 2012. The latest treatment resulted in recovery from symptoms and a more stable condition. However, cough and sputum production, although attenuated, persisted despite treatment and negative microbiological test results. High-resolution CT images (Figure, panel C) indicated overall progression of pulmonary involvement from the time of first admission.

In February 2011, on the basis of the known cross-reactivity of the *M. intracellulare* probe with most MAC species when the GenoType Mycobacterium CM/AS assay is used (*3*), we determined the sequences of a portion of the *rpo*B gene and internal transcribed spacer–1 region in 2 strains isolated from sputum in March 2006 and June 2010. All sequences overlapped with *M. marseillense* type strain sequences in GenBank, showing an identity of 100% in internal transcribed spacer–1 with EU266631 and 99.8% (1 mismatch) in *rpo*B with EF584434.

We therefore show the association of *M. marseillense* infection with pulmonary disease in an immunocompetent patient, helping define the clinical features and natural history of pulmonary disease caused by *M. marseillense*. We cannot assert whether the clinical course is associated with the intrinsic characteristics of *M. marseillense* infection or with the therapeutic regimen, possibly influenced by numerous adverse effects that may have compromised its effectiveness. More careful management in accordance with the American Thoracic Society and the Infectious Diseases Society of America guidelines for management of nontuberculous mycobacterial diseases could have achieved a more effective course of treatment. More case reports of pulmonary disease caused by *M. marseillense* are needed to support our observations and to provide more insight into its clinical picture.

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Usutu Virus in Bats, Germany, 2013

To the Editor: Usutu virus (USUV) is an arthropod-borne flavivirus that belongs to the Japanese encephalitis serocomplex (*1*). USUV circulates between ornithophilic mosquito vectors (mainly *Culex* spp. mosquitoes) and avian amplification hosts (*2*). Migratory birds play a key role in the introduction of USUV into new areas (*3*). USUV has recently been introduced from Africa into Europe, causing epizootics among wild birds and Usutu fever in humans (*4*–*6*). The detection and isolation of USUV from different bird and mammalophilic mosquitoes during the epizootic in Germany raise questions regarding the USUV host range (*2*,*3*). Bats have been considered natural reservoir hosts of a wide diversity of viruses, including several flaviviruses (*7*,*8*). Their ability to fly and their social behavior enable efficient maintenance, spread, and evolution of viruses.

In September and October 2013, in southwest Germany, 2 dead bats were found within ≈15 km of each other (bat 1, Ludwigshafen am Rhein, 49°28′34″N 8°26′46″E; bat 2, Waldsee, 49°23′44″N 8°26′27″E), corresponding to the previously described USUV-endemic area (*2*,*3*). A full necropsy was conducted on each bat, and samples were collected for virus detection, histologic analysis, and bat species determination.

Total DNA and RNA were extracted from tissue samples (brain, liver, lung, and heart) and subjected to reverse transcription PCR for rhabdovirus and flavivirus (*2*). Histologic analysis of the tissue samples was not successful because of autolysis. Use of a cytochrome b–specific PCR and direct sequencing of the PCR amplicons genetically identified each bat as a common pipistrelle (*Pipistrellus pipistrellus*) (*9*).

The bat samples were negative for rhabdoviruses but positive for flaviviruses (brain tissue only). Direct sequencing of the PCR amplicons revealed that the USUV sequences were related to the recently described bird-derived USUV strain BH65/11–02–03 from Germany (*2*). Attempts to isolate the bat USUV strains in cell culture were not successful, probably because of autolysis. However, the complete genome sequences of both bat USUV strains (BAT1USUTU-BNI, KJ859682; BAT2USUTU-BNI, KJ859683) were then determined directly from the brain samples by using primers (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/10/14-0909-Techapp1.pdf) designed from multiple alignments of USUV genomes obtained from databases.

The 2 genomes had an identical size of 11,065 nt with a 96-nt 5′ nontranslated region and a 664-nt 3′ nontranslated region. The single open reading frame encodes a polyprotein of 3,434 aa. Both bat USUV strains had 99.9% nt and 99.8% aa identity. We compared the 2 bat USUV strains with those detected in mosquitos, birds, and humans from Europe and Africa; the sequence identities of nucleotides varied from 78.3% to 99.3% and of amino acids from 90.8% to 99.3%. The sequence identity matrix with the USUV strain BH65/11–02–03 from Germany was 99.3% for nucleotides and 99.2% for amino acids. Comparison of the *Pipistrellus* bat USUV complete polyprotein sequence with mosquito and bird-derived strains showed 2 aa substitutions—one (A1236V) in the nonstructural protein (NS) 2a and the other (L1549F) in the NS3 gene—which were detected only in the bird-derived USUV strain BH65/11–02–03 from Germany. In addition, 2 additional unique amino acid substitutions (A1841V and K1870M) in the NS3 protein gene of the BAT1USUTU-BNI strain were also identified. Bayesian and maximum-likelihood phylogenetic analyses of the full-length sequences revealed the close relationship of the *Pipistrellus* bat–derived USUV strains with the 2011 bird-derived strain BH65/11–02–03 from Germany, forming a distinct group within the phylogenetic tree (group Europe 3) (Figure). A partial envelope and NS5-gene–based phylogenetic analysis that used more available sequences from databases yielded the same topology (data not shown).

Pipistrellus bats are highly prevalent in Germany. Their geographic range overlaps with that of the USUV epizootic. Thus, considerable interactions between birds, mosquitoes, and bats could have occurred. The amino acid replacements (A1236V and L1549F) detected in the NS genes of *Pipistrellus* bat–derived USUV strains and the bird-derived USUV strain from Germany suggest an adaptive evolution, which probably occurred during the introduction of the virus into Germany.

Although the role of these mutations is not known, similar mutations in the related West Nile virus modulated the host antiviral response by inhibition of interferon signaling (*10*). Our results suggest that bats probably contribute to the epizootic rather than act as a silent reservoir for the virus. In contrast, infections of bats might be merely coincidental to what may well be broader infections of vertebrates in the epizootic area. However, for confirmation of this hypothesis, further investigations are required. Although the detected bat-derived sequences are somehow distinct from sequences of other USUV strains, a spillover infection from birds or another, yet unrecognized, host cannot be ruled out. The detection of the virus exclusively in brain tissue suggests that USUV might have a higher tropism for the nervous system in bats, as opposed to the pantropism observed in birds (*2*). The detection of USUV in bats raises questions for further research, including the potential role of bats as reservoirs in Africa and transmission by mosquito vectors.

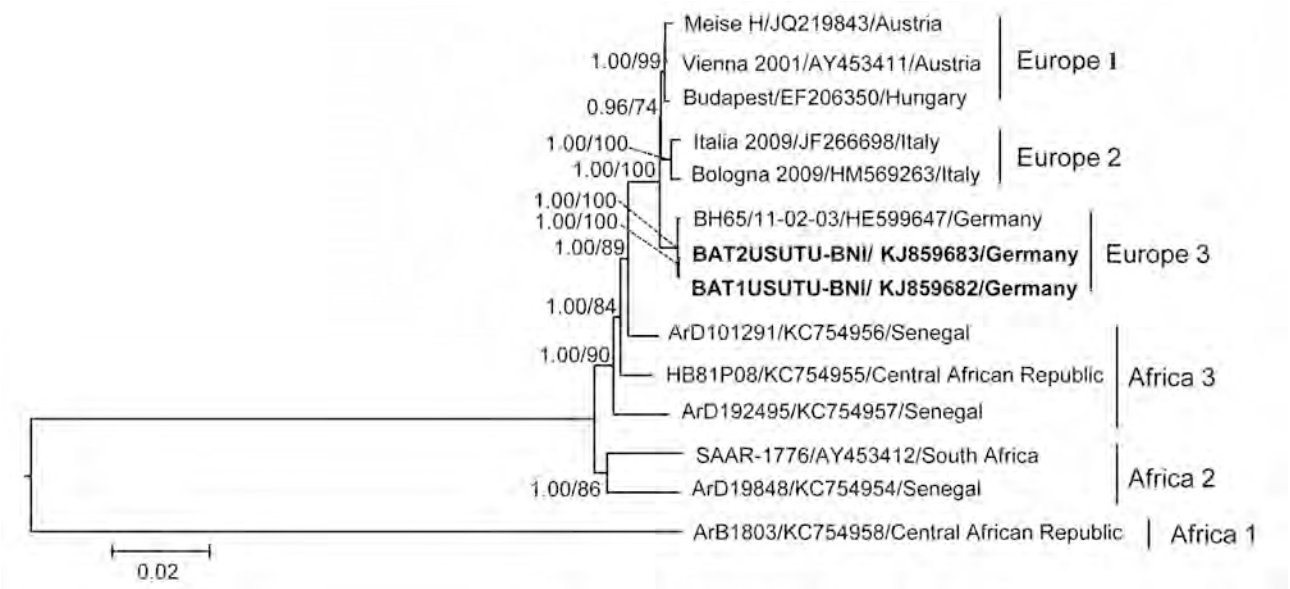

Figure. Maximum-likelihood phylogenetic tree of *Pipistrellus* bat Usutu viruses (USUV) based on full-length nucleotide sequences and showing the phylogenetic placement of the bat-derived USUV compared with human-, mosquito-, and bird-derived strains. The phylogenetic analyses were performed by using PhyML 3.0 (http://www.atgc-montpellier.fr/phyml/versions.php) with 1,000 pseudo-replicates and parallel Bayesian Markov chain Monte Carlo tree-sampling methods based on 2 runs consisting of 4 chains of 1,000,000 with a burn-in of 25% using MrBayes 3.1.2 (http://mrbayes.sourceforge.net/). The Akaike information criterion was chosen as the model selection framework and the general time-reversible model of sequence evolution with gamma-distributed rate variation among sites as the best model. Numbers at the nodes indicate maximum-likelihood bootstrap replicates (≥70%) and parallel the posterior probability values (clade credibilities ≥90%). Boldface indicates USUV strains from *Pipistrellus* bats in Germany in 2013 (this study). Strain names, GenBank accession numbers, and countries of origin for sequences used to construct the tree are indicated on the branches. Scale bar indicates mean number of nucleotide substitutions per site.

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We dedicate this letter to the late Ursula Herrmann (1927–2014), who made this study possible.

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Chikungunya Virus Imported into French Polynesia, 2014

To the Editor: Chikungunya virus (CHIKV) is an emerging arthropodborne alphavirus of the family *Togaviridae* (*1*). The most common clinical manifestations of infection with CHIKV are abrupt onset of fever, headache, back pain, myalgia, and arthralgia affecting mainly the extremities; in ≈50% of patients, a rash develops, and relapsing and incapacitating arthralgia is common (*1*). Three CHIKV lineages have been characterized: West African, Asian, and East/Central/South African (*1*,*2*). The strain currently circulating in the Caribbean belongs to the Asian lineage (*2*).

In the Pacific region in 2011, a CHIKV outbreak was reported in New Caledonia (*3*). Additional outbreaks have been reported in Papua New Guinea in June 2012 (*4*), Yap State in August 2013 (*5*), and in the Kingdom of Tonga in April 2014 (*6*). In the Caribbean region, cases of CHIKV infection were reported in the French part of Saint Martin Island in December 2013, after which CHIKV rapidly spread to other Caribbean islands, including Guadeloupe (*2*), where by the end of May 2014 it had caused an estimated 23,100 infections.

On May 25, 2014, a healthy 60-year-old woman returned to French Polynesia after a 6-month stay with her husband's family in Guadeloupe, where she had been in contact with family members who reportedly had chikungunya. On the first night after arriving back home in French Polynesia, she noted headache, transient high fever, and mild arthralgia of the knees. The next day, she sought care from her general practitioner for weakness, headache, and severe polyarthralgia (wrists, fingers, knees, toes). Physical examination revealed only swollen fingers and toes; the patient was not febrile. Blood samples were collected, and the patient was administered acetaminophen and corticosteroids. Her headache persisted until day 3, and arthralgia persisted until day 4.

Laboratory tests revealed lymphopenia (589×10^6 cells/L) and slightly elevated C-reactive protein (14.2 mg/L) and liver enzyme levels (aspartate aminotransferase 44 IU/L, gamma-glutamyl transferase 58 IU/L). CHIKV infection was confirmed by a specific real-time reverse transcription PCR (rRT-PCR) with previously reported primers and probe (*7*) and by partial sequencing of the E1 gene (GenBank accession no. KJ939333). Phylogenetic analysis (Figure) showed that the virus strain isolated from the patient was most closely related to strains isolated in the British Virgin Islands in 2014 (VG14/99659, accession no. KJ451624), Yap State, Federated States of Micronesia, in 2013 (FM13/3807, accession no. KJ451622), and Zhejiang Province, China, in 2012 (CN12/chiksy, accession no. KF318729), with 100%, 99.89%, and 99.78% homology, respectively, thereby confirming its inclusion in the Asian lineage. A blood sample from the patient was inoculated into Vero and *Aedes albopictus* C6/36 cells. Cells were incubated for 6 days, after which time both supernatants were removed and tested. RT-PCR, as described above, gave positive results for CHIKV.

After CHIKV infection was confirmed, the case was immediately reported to health authorities in French Polynesia. Vector control measures were immediately implemented and included individual protection against mosquito bites (mosquito repellents) for the patient and her close social and family contacts and collective protection (insecticide spraying and breeding site elimination) targeting the house of the patient and the areas that she had visited. Written informed consent was obtained from the patient before publication of this case report.

Arbovirus diseases are endemic to French Polynesia. Dengue virus serotypes 1 and 3 have been cocirculating since 2013 (*8*); and, from October 2013 through April 2014, a large outbreak of Zika virus infection occurred (*9*). Because this case provides evidence of the possible emergence of CHIKV in French Polynesia, health authorities and health care workers in French Polynesia were immediately alerted and prepared to detect local transmission of CHIKV infection.

CHIKV is transmitted by mosquitoes of the *Aedes* species, especially *Ae. aegypti* and *Ae. albopictus* (*1*,*2*). The risk for emergence of a chikungunya outbreak in French Polynesia is high because of the presence of 2 potential vectors: *Ae. aegypti* mosquitoes, vectors of CHIKV in New Caledonia (*3*) and in Guadeloupe (*2*), and *Ae. polynesiensis* mosquitoes, potential CHIKV vectors as suggested by experimental infections (*10*).

The role of foreign travel in spreading arboviruses between French overseas territories is highlighted by the observation that the CHIKV-infected patient reported here returned from Guadeloupe and by a previous report that the 2013 outbreak of dengue virus type 3 in French Polynesia was caused by a virus introduced from French Guiana (*8*). Zika fever was reported in French Polynesia in October 2013; within the next 6

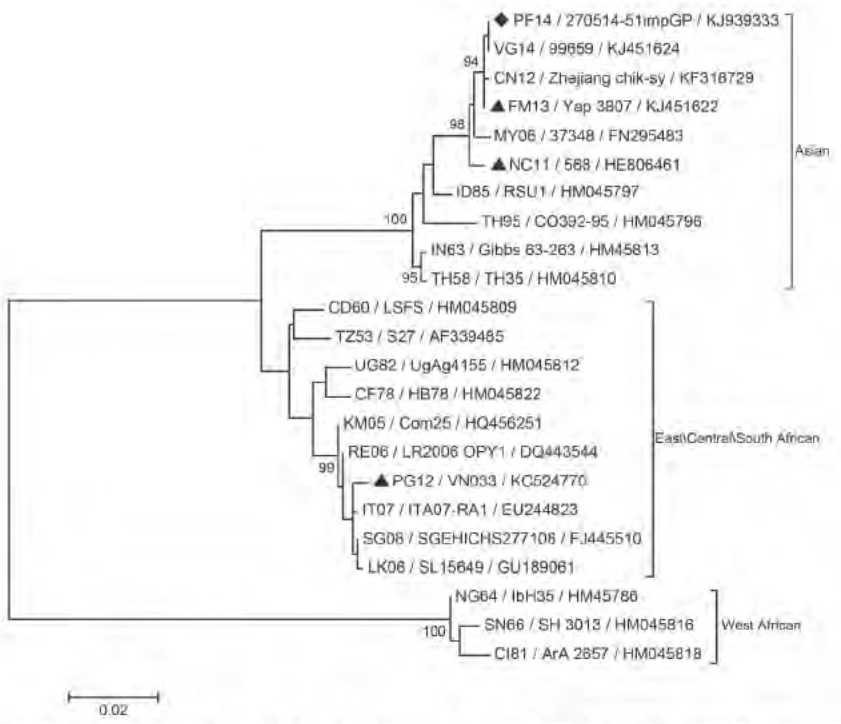

Figure. Phylogenetic analysis of the chikungunya virus strain isolated from a patient in French Polynesia, May 2014. The evolutionary history was inferred by using the maximum-likelihood method based on the Kimura 2-parameter model. The percentage of trees in which the associated taxa clustered together is shown for values >90 next to the branches (1,000 replicates). Evolutionary analyses were conducted by using MEGA software, version 6 (http://www.megasoftware.net). Each strain is labeled by country (International Organization for Standardization 2-letter country codes, http://www.iso.org/iso/home/standards/country_codes.htm) and date of origin /strain name/GenBank accession number. Black diamond indicates the chikungunya virus strain from French Polynesia; black triangles indicate strains from other Pacific islands. Scale bar indicates number of substitutions per site.

months, 28,000 suspected cases were reported (*9*), but the index patient was not identified.

As soon as this case of chikungunya in French Polynesia was reported, control measures were applied; as of 4 weeks later, no autochthonous cases have been reported. However, because the French Polynesia population has not been exposed to CHIKV, as it had not been exposed to ZIKV, the possibility of evolution toward a large CHIKV outbreak cannot be excluded. Reinforced epidemiologic surveillance and laboratory capacities are needed for determining whether chikungunya will extend further in French Polynesia and in the Pacific region.

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Probable Importation of Dengue Virus Type 4 to Angola from Brazil

To the Editor: The prevalence of dengue virus (DENV) infection in Africa may have been grossly underestimated for many years (*1*). Even though published reports are scarce, dengue has been documented in at least 34 countries in Africa (*2*). The role of travelers as sentinels of infectious disease has proven to be invaluable in this regard; dengue in returning travelers was the only evidence for local DENV transmission for 12 of these countries.

During April 2013, simultaneous reports of travelers returning from Luanda, Angola, with dengue fever emerged from Israel, several countries in Europe, Canada, and South Africa; PCR and sequencing of viral RNA confirmed the causative species to be DENV type 1 (DENV-1) (*3*). Concomitantly, a large outbreak of dengue was confirmed by local authorities in Luanda, and DENV-1 was isolated in samples from local residents (*4*). The origin of the outbreak strain was not ascertained, but phylogenetic analysis suggested that it had close affinity with isolates from West Africa (*4*,*5*). These reports involved almost 100 travelers and >500 residents of Luanda who become within a short time. Active DENV transmission and occurrence of new cases in Luanda were still ongoing during early July 2013 (*6*), but since then, to our knowledge, no additional local data on DENV activity in Luanda have been published.

We report 3 new cases of dengue fever acquired in Luanda during December 2013–February 2014. Two cases occurred in travelers who returned to Israel, and 1 occurred in a traveler who returned to Germany. All 3 cases occurred in middle-aged businessmen who had traveled to Angola and who were hospitalized within days of returning to their home countries because of signs and symptoms of dengue fever. None of the case-patients met criteria for severe dengue, and all recovered uneventfully. Acute DENV infection was confirmed by serologic testing in all 3 patients and by positive results for a nonstructural protein 1 serum antigen test in 2 travelers.

In a serum sample taken from 1 of the travelers from Israel on the second day of fever, DENV RNA was detected by using reverse transcription PCR. This strain was found to belong to DENV type 4 (DENV-4). Phylogenetic analysis was performed, and multiple sequence alignment of this DENV-4 sequence, in comparison to other DENV-4 sequences retrieved from GenBank, was performed by using the Sequencher 5.0 program (Gencodes Corporation, Ann Arbor, MI, USA). A maximum-likelihood phylogenetic tree was inferred from the sequence alignment by using ClustalX (http://www.clustal.org), and the robustness of the tree was assessed by 1,000 bootstrap replications. The tree was visualized and produced by using NJ plot software (http://doua.prabi.fr/software/njplot). Results showed that RNA from this isolate was most closely related to that of a DENV-4 strain identified in 2010 in Boa Vista, the capital of Roraima State in the Amazon Region of Brazil (*7*) (Figure).

DENV-4 infection was previously reported in Africa in 1986, when 2 cases were identified in travelers returning from Senegal (*8*), but to our knowledge, no other cases have been reported in western or southwestern Africa. During the April 2013 dengue outbreak in Luanda, only DENV-1 was isolated (*3*,*4*). After that outbreak, a study conducted on the basis of modeling of international commercial flight data to and from Angola suggested that DENV would most likely have been imported from Latin America (*5*). Our finding, a year later, of DENV-4 in Angola that was closely related to strains from Brazil appears to vindicate this modeling system. During the past decade, Brazil had been experiencing a consistent increase in dengue epidemics and in severity of disease (*9*). Brazil's large and growing economy, its increasingly prominent place in world trade, and its growing tourism industry high-

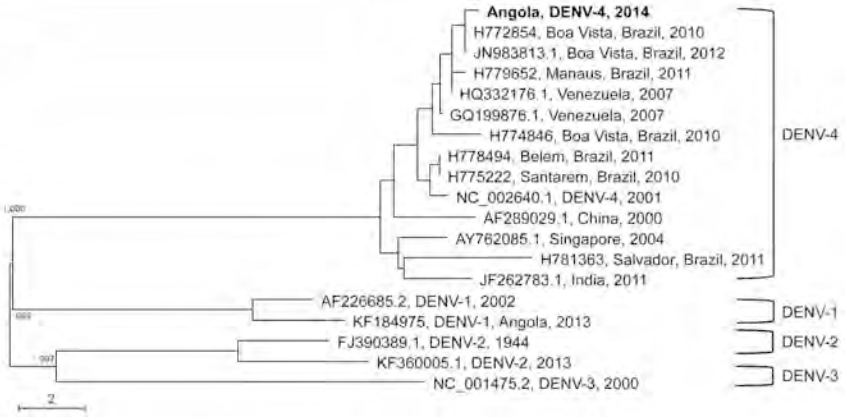

Figure. Phylogenetic analysis of a dengue virus (DENV) type 4 strain isolate (boldface) from a patient in Israel who apparently acquired DENV in Angola in 2014, showing close relationship with isolates from Brazil. The DENV isolate was aligned with representative DENV sequences from around the world, representing serotypes 1–4. Reference strains, downloaded from public databases (http://www.ncbi.nlm.nih.gov/nuccore), are identified by accession number, place, and year of isolation (DENV-4 isolates) or by serotype, accession number, and year of isolation (DENV-1–3 isolates). Specific branches indicate bootstrap values. Scale bar indicates percentage identity difference.

light the country's potential role in the global circulation of DENV.

Our findings are corroborated by a recently reported case of dengue in a traveler from Portugal that was acquired in Luanda concomitantly with our cases and also found to be caused by DENV-4 (*10*). In light of the apparent introduction of DENV-4 to Angola, probably from Brazil, health authorities should be encouraged to enhance surveillance and vector control efforts. In addition, health practitioners treating travelers returning from Angola should be aware of the risk for DENV infection.

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Borrelia garinii and *Rickettsia monacensis* in *Ixodes ricinus* Ticks, Algeria

To the Editor: Lyme disease (Lyme borreliosis) is caused by a group of related spirochetes (*Borrelia burgdorferi* sensu lato) that include ≥11 species (*1*). In northern Africa, the main vector of Lyme disease in Europe (*Ixodes ricinus* ticks) is also present, and this disease has been suspected to be present in this region of Africa (*2*). Twenty-one cases of Lyme disease were reported in Algiers, Algeria, during 1996–1999 (*3*). However, these cases were diagnosed by detection of only serum antibodies against *B. burgdorferi* by ELISA without confirmation by Western blotting.

I. ricinus ticks are also known to harbor spotted fever group rickettsiae, including *Rickettsia monacensis*, which was detected in Algeria in 2009 (*4*). This rickettsia has been recently identified as a human pathogen in Spain and Italy (*5*).

To investigate Lyme disease and tickborne rickettsioses transmitted by *I. ricinus* ticks in northeastern Algeria, we collected ticks by using the flag method in El Ghora (Bougous, El Tarf) (36°39′34″N, 8°22′10″E). Ectoparasites were collected in March 2012 and identified to genus and species by using taxonomic morphologic keys (*6*).

Total genomic DNA was isolated by using the QIAamp Tissue Kit (QIAGEN, Hilden, Germany) and BioRobot EZ1 (QIAGEN) as described by the manufacturer. DNA was used as template for quantitative real-time PCR. We used the RKND03 system, which is specific for the *gltA* gene of *Rickettsia* spp. (*7*), and the Bor16S system, which is specific for the *rrs* gene of *Borrelia* spp. (*8*). Real-time PCRs were performed by using the CFX96 Real Time System C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Singapore).

Positive results were confirmed by using a standard PCR specific for the *ompA* gene of *Rickettsia* spp. and the 16S rRNA and *flaB* genes of *Borrelia* spp. (*8*). We used bacteria-free DNA of *Rhipicephalus sanguineus* ticks reared in our laboratory colonies as a negative control and DNA of *B. crocidurae* and *R. montanensis*, which are not known to be associated with *I. ricinus* ticks, as a positive control.

PCR amplification was verified by electrophoresis of products on 2% agarose gels. Products were purified by using a NucleoFast 96 PCR plate (Macherey-Nagel EURL, Hoerdt, France) as recommended by the manufacturer. Purified PCR products were sequenced by using the same primers as for a standard PCR and the BigDye version 1–1 Cycle Ready Reaction Sequencing Mixture (Applied Biosystems, Foster City, CA, USA) in the ABI 31000 automated sequencer (Applied Biosystems). Sequences were assembled and analyzed by using ChromasPro version 1.34 software (Technelysium Pty. Ltd., Tewantin, Queensland, Australia).

Ninety-four ticks were collected by using the dragging method; these ticks belonged to 2 species: 85.1% (80/174) were *I. ricinus* ricks (43 females, 22 males, and 15 nymphs) and 14.9% (14/94) were *Rh. sanguineus* adult ticks (11 females and 3 males). We screened only the 80 *I. ricinus* ricks. *Rh. sanguineus* ticks were kept alive to establish laboratory colonies for other experiments. Overall, 5.0% (4/80) of *I. ricinus* ticks were positive for *Borrelia* spp. and 8.75% (7/80) were positive for *Rickettsia* spp.

Using a standard PCR specific for the *flaB* gene, we identified *B. garinii* in all ticks positive by quantitative real-time PCR (100% similarity, 736/736 bp) (GenBank accession no. CP003151.1). Using a standard PCR specific for the *ompA* gene of *Rickettsia* spp., we identified *R. monacensis* (100% similarity 760/760 bp) (GenBank accession no. FJ919640.1).

We have detected *B. garinii*, a cause of Lyme disease, in Algeria in *I. ricinus* ticks by using a standard PCR and sequencing methods. We also confirmed the presence of *R. monacensis* in this country.

Borrelia spp. have been detected in *I. ricinus* ticks in Tunisia and Morocco (*2*,*9*), and *B. lusitaniae* was found to be predominant (97% of *Borrelia* spp. in Tunisia and 93% in Morocco). However, *B. garinii* was also present (*2*,*9*,*10*). In Tunisia, 1/16 *I. ricinus* ticks were positive for *B. garinii* (*2*,*9*). In Morocco, 3 (3.6%) of 82 were positive for *B. burgdorferi* sensu stricto and 3 (3.6%) of 82 were positive for *B. garinii* (*9*). However, in these studies, *Borrelia* spp.were identified by using restriction fragment length polymorphism analysis (*2*,*9*).

B. garinii is the most neurotropic of the genospecies of *B. burgdorferi* sensu lato; it causes meningopolyneuritis and, rarely, encephalomyelitis (*1*). Clinicians need to be aware of the prevalence of this bacterium in Algeria. Our results help clarify the epidemiology of *B. garinii* in Algeria. *R. monacensis* is an agent of tickborne diseases that was detected in Algeria in 2009 (*4*). The few cases that have been described were characterized by influenza-like symptoms, fever, an inoculation eschar, and a generalized rash (*5*).

In northern Africa, the risk areas for Lyme disease and infection with *R. monacensis* include cool and humid areas in the Atlas Mountains. In this region, humans can come in contact with *I. ricinus* ticks, and these ticks might play a major role in transmission of *B. garinii* and *R. monacensis*.

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Imported Malaria in China, 2012

To the Editor: Imported malaria has become a major public health challenge in China. Despite an 89.8% decrease in total cases from 2008 (26,873) through 2012 (2,729), the proportion of imported malaria cases has increased from 14.7% to 89.0% (*1*). We analyzed the malaria situation in China in 2012 by using data obtained from the national information reporting system of infectious diseases.

In this analysis, an imported case of malaria was defined as case of malaria acquired in a known malarious area outside China. In China, the following criteria for imported malaria must be simultaneously met: 1) the patient was given a diagnosis of malaria; 2) the patient had a travel history to malaria-endemic areas outside China during malaria transmission season; and 3) the onset time for malaria for the person was <1 month after returning to China during the local transmission season. This definition of malaria was based on the latent period for all *Plasmodium* species reported in China.

Of 2,729 malaria cases reported in 2012, a total of 2,428 (89.0%) were imported. Fifteen of these cases were in persons who died of infection with *P. falciparum*. In the imported cases, 4 *Plasmodium* species were identified: *P. falciparum* (n = 1,423 [58.6%]), *P. vivax* (n = 909 [37.5%]), *P. ovale* (n = 42 [1.7%]), *P. malariae* (n = 20 [0.8%]), and mixed infections (n = 21 [0.9%]). Among imported cases, 13 (0.5%) were clinically diagnosed.

Chinese workers who returned from Africa (n = 1,458 [60.0%]) had most cases imported malaria. A total of 37 countries in Africa were sources of imported cases. Most cases were acquired in Ghana (n = 241 [9.9%]), Equatorial Guinea (n = 233 [9.6%]), and Nigeria (n = 197 [8.1%]). Case-patients were predominantly infected by *P. falciparum* (n = 1,187 [81.4%]). Southeast Asia (n = 895 [36.9%]), including Myanmar (n = 764 [31.5%]), Cambodia (n = 49 [2.0%]), and Laos (n = 36 [1.5%]), was another major source of imported cases. These case-patients were infected mostly with *P. vivax* (n = 658 [73.5%]) (Table).

Imported cases increased during April, reached a peak during May (n = 297 [12.2%]), and decreased during July. This trend was caused by workers returning to China to perform agricultural work during this period. The male:female patient ratio was 14.6:1 (n = 2,272 male patients:156 female patients); most (n = 2,240 [95.2%]) mobile laborers are men. The mean age of persons with imported cases was 40.8 years. Most (n = 1,813 [74.7%]) of these persons were 15–44 years of age and few (n = 5 [0.2%]) were <5 years of age.

Persons with imported cases were detected in 29 provinces (Hong Kong, Macao, and Taiwan did not join the information system). Yunnan (n = 690 [28.4%]), Guangxi (n = 209 [8.6%]), and Jiangsu (n = 197 [8.1%]) Provinces had the largest number of imported cases.

Our analysis indicated that imported malaria poses major challenges to the malaria elimination program in China. One challenge is the increasing investment in overseas work and increasing numbers of Chinese persons who are working abroad. The total number of Chinese laborers and travelers abroad in 2012 was estimated to be 0.5 million and 83.2 million persons, respectively; these numbers increased by 24.6% and 44.9%, respectively, from numbers in 2010. Another reason for the increasing proportion of imported malaria cases was a sharp decrease in locally acquired infections. There were only 246 locally acquired cases in 2012, a decrease of 94.2% from the number of locally acquired cases in 2010 (*2*).

Because imported malaria is widely distributed throughout China, the disease could be introduced into malaria-free localities during the transmission season, especially when a large number of cases are clustered in areas in which *Anopheles* species mosquitoes are prevalent. Additional studies are needed to determine the susceptibility of *Anopheles* species mosquitoes in China to *Plasmodium* species that cause human malaria.

In summary, imported malaria poses a severe threat to the malaria elimination program in China (*3*). For effective management of imported malaria, surveillance systems need to be carefully planned and well managed to ensure timely recognition and prompt response. Effective mechanisms of multisectoral coordination and cooperation should be established and strengthened. In addition, health education information on malaria risks and protection should be provided to all mobile laborers and other travelers before their traveling abroad and after returning home. Labor and travel agencies should provide travelers with essential preventive measures. This information should also be provided to entry and exit border stations and to local Centers for Disease Control and Prevention so that timely malaria tracking can be implemented. Training should also be provided to physicians to ensure provision of accurate diagnosis and appropriate treatment. For local health agencies, prompt case verification and response are required to ensure elimination of residual potential reservoirs and prevention of local transmission caused by imported pathogens.

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Table. Malaria cases imported into China from other countries, by country and *Plasmodium* species, 2012

Country of origin	Total, n = 2,428, no. (%)	Species, no. (%)					
		P. falciparum, n = 1,423	*P. vivax*, n = 909	*P. malariae*, n = 20	*P. ovale*, n = 42	Mixed, n = 21	Unclassified, n = 13
Africa	1,458 (60.0)	1,187 (83.4)	192 (21.1)	16 (80.0)	40 (95.2)	12 (57.1)	11 (84.6)
Ghana	241	207	21	4	2	3	4
Equatorial Guinea	233	187	25	2	15	3	1
Nigeria	197	172	19	1	5	0	0
Angola	151	127	13	4	5	0	2
Guinea	60	56	3	0	1	0	0
Sudan	50	34	16	0	0	0	0
Liberia	45	32	10	1	1	1	0
Republic of Congo	43	34	4	1	2	2	0
Sierra Leone	42	36	3	0	1	1	1
Gabon	37	31	4	0	2	0	0
Democratic Republic of Congo	36	28	6	0	1	1	0
Ethiopia	30	9	21	0	0	0	0
Mozambique	20	18	2	0	0	0	0
Cameroon	18	12	6	0	0	0	0
Côte d'Ivoire	17	17	0	0	0	0	0
Mali	17	15	2	0	0	0	0
Tanzania	17	15	2	0	0	0	0
Zambia	16	15	1	0	0	0	0
Uganda	16	11	4	0	1	0	0
South Sudan	16	16	0	0	0	0	0
Chad	11	8	3	0	0	0	0
Malawi	10	8	1	0	1	0	0
Central African Republic	8	7	0	1	0	0	0
South Africa	8	4	2	1	1	0	0
Senegal	5	4	1	0	0	0	0
Benin	5	4	1	0	0	0	0
Burkina Faso	5	5	0	0	0	0	0
Kenya	5	4	1	0	0	0	0
Madagascar	5	5	0	0	0	0	0
Niger	4	4	0	0	0	0	0
Libya	3	1	2	0	0	0	0
Zimbabwe	2	2	0	0	0	0	0
Togo	2	2	0	0	0	0	0
Rwanda	2	1	1	0	0	0	0
Mauritania	1	0	1	0	0	0	0
Egypt	1	1	0	0	0	0	0
Algeria	1	0	1	0	0	0	0
Unknown	78	55	16	1	2	1	3
Southeast Asia	895 (36.9)	224 (15.7)	658 (72.4)	3 (15.0)	1 (2.4)	9 (42.9)	0
Myanmar	764	198	557	2	1	6	0
Cambodia	49	1	46	1	0	1	0
Laos	36	4	32	0	0	0	0
Indonesia	35	17	16	0	0	2	0
East Timor	3	0	3	0	0	0	0
Vietnam	5	3	2	0	0	0	0
Thailand	1	0	1	0	0	0	0
Malaysia	1	1	0	0	0	0	0
Unknown	1	0	1	0	0	0	0
Southern Asia	45 (1.8)	4 (0.3)	39 (4.3)	0	1 (2.4)	0	1 (7.7)
Pakistan	29	4	24	0	1	0	0
India	14	0	13	0	0	0	1
Afghanistan	2	0	2	0	0	0	0
Eastern Asia	2 (0.1)	0	2 (0.2)	0	0	0	0
South Korea	1	0	1	0	0	0	0
North Korea	1	0	1	0	0	0	0
Oceania	6 (0.2)	2 (0.2)	4 (0.5)	0	0	0	0
Papua New Guinea	6	2	4	0	0	0	0
Latin America	1 (0.1)	0	1 (0.1)	0	0	0	0
Brazil	1	0	1	0	0	0	0
Unknown	21 (0.9)	6 (0.4)	13 (1.4)	1 (5.0)	0	0	1 (7.7)

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Two Human Cases of *Rickettsia felis* Infection, Thailand

To the Editor: *Rickettsia felis* is an emerging pathogen responsible for fleaborne spotted fever. This new species was first isolated in 2002 from the cat flea, *Ctenocephalides felis*, which was then identified as the main vector of this rickettsia (*1*). *R. felis* has also been described in other flea, tick, chigger, and mite species (*2*) and more recently, in mosquitoes, which are strongly suspected to play a role in transmission of the bacterium (*3,4*).

The first evidence of human pathogenicity of *R. felis* was suspected in a patient from Texas, USA, in 1991 and was confirmed by 3 additional case-patients from Mexico in 2000 (*5*). Evidence suggests that this bacterium has a worldwide distribution; ≈100 reports of human cases have been published (*2*,*6*). Moreover, *R. felis* was identified as a common (3%–15%) cause of fever among febrile patients in tropical Africa (*7*). The bacterium has also been described in Asia, but little is known about cases of infection in humans; only 1 human case was described in Thailand in 2003 (*8*).

We enrolled febrile patients (≥7 years of age) who came to 4 community hospitals, 2 in Chiang Rai (northern Thailand) and 2 in Khon Kaen (northeastern Thailand) during 2002–2005. Acute-phase and convalescent-phase (3–5 weeks later) serum samples were obtained from 2,225 patients and tested for *R. felis* by using an indirect immunofluorescence assay (*9*). Seventeen (0.8%) of 2,225 patients showed evidence of seroconversion (IgG titer ≥1:128 or IgM titer ≥1:64 or a ≥4-fold increase in titer).

Specific real-time PCR (qPCR) for *R. felis* was performed with acute-phase serum samples of these patients with primers and probes specific for *orfB* and *vapB1* genes as described (*7*). DNA was extracted by using the Biorobot EZ1 Workstation (QIAGEN, Courtaboeuf, France), and qPCR was performed by using a CFX96 instrument (Bio-Rad, Marne-la-Coquette, France). DNA from *R. felis* strain URRWFXCal^T (*1*) was used as a positive control, and sterile water was used a negative control. The qPCR results were positive (cycle threshold ≤35) for the 2 genes for 2 of the 17 patients; the four 150-bp amplicons were sequenced. Sequences of *orfB* (150/150) and *VapB1* (155/155) showed 100% similarity with the sequence from the complete genome of *R. felis* URRWXCAL^T (GenBank accession no. CP000053).

Patient 1, a 20-year-old woman, and patient 2, a 45-year-old man, were from Chiang Rai Province. They both had fever, myalgia, arthralgia, headache, abdominal pain, cough, and chest pain. No rashes, eschars, or lymphadenopathies were noted. In addition, patient 2 had photophobia, had vomited, and reported contact with cats. Both patients reported having contact with others animals and being bitten by insects, including mosquitoes (Table).

R. felis DNA was detected in serum samples from these 2 patients with acute febrile illness in Thailand. The immunofluorescent assay, the reference serologic method for diagnosis of infection with *Rickettsia* spp., is known to show cross-reactivity with other *Rickettsia* spp. Therefore, diagnosis of rickettsial infection should be confirmed by Western blotting or molecular testing. Real-time PCRs are increasingly being used for diagnosis of rickettsioses, including those with *R. felis*, and for vector and reservoir identification (*2*).

The predominant rickettsioses reported in Asia are murine typhus and scrub typhus, which are caused by *R. typhi* and *Orientia tsutsugamushi*, respectively (*8*). To the best of our knowledge, only 12 human cases of *R. felis* infection have been reported in Asia: 3 in Thailand (including these cases), 3 in Sri Lanka, 1 in Laos, 1 in Israel, 1 in Taiwan, and 3 in South Korea (*2*,*8*–*10*). The prevalence of *R. felis* in fleas has been well studied in >20 countries, including Japan, Thailand, Indonesia, Laos, Taiwan, Israel, Afghanistan, and Lebanon (*2*). This bacterium has also been described in mites in Taiwan and South Korea, in

Table. Characteristics of 2 febrile patients with confirmed *Rickettsia felis* infection, Chiang Rai Province, Thailand*

Characteristics	Patient 1	Patient 2
Age, y	20	45
Sex	F	M
Acute-phase IgG/IgM titer	0/0	0/0
Convalescent-phase IgG/IgM titer	128/32	128/0
Hospitalization	Yes	Yes
Animal exposure within past 2 wk before illness		
Cats	No	Yes
Dogs	Yes	Yes
Rodents	No	No
Insect bites	Yes	Yes
Mosquito bites	Yes	Yes
Laboratory values		
Leukocytes/mm^3	6,300	4,100
Hemoglobin, g/dL	11.7	16.0
Platelets/mm^3	103,000	216,000
BUN, mg/dL	62	31.1
Creatinine, mg/dL	3.56	1.1
ALT, IU/dL	44.6	29.1
AST, IU/dL	49.8	30.2
Bilirubin, mg/dL	0.5	0.97
Alkaline phosphatase, IU/dL	132	137

*BUN, blood urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

chiggers in South Korea, and in ticks in Japan (*2*,*9*,*10*).

The clinical signs and symptoms of *R. felis* infection are now better understood. The more frequent clinical findings reported are nonspecific and include fever, asthenia, headache, maculopapular rash, and inoculation eschar. Neurologic, digestive, and respiratory symptoms are not commonly reported (*2*). These infections could be confused with other rickettsioses or other febrile illnesses, such as malaria. In most regions, laboratory tests are unavailable; consequently, *R. felis* infections are largely underdiagnosed.

The findings of this study indicate that *R. felis* infections may be among the causes of febrile illness in Thailand and highlight the need for physicians to consider this pathogen in the differential diagnosis of diseases in tropical countries and in travelers. Further studies are needed to ascertain risk factors and confirm the causal association and pathology of fleaborne spotted fever in Asia.

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Emerging Infections: A Guide to Diseases, Causative Agents, and Surveillance

Lisa A. Beltz

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Given the importance of emerging infectious diseases, it is surprising that there are few books dedicated solely to this subject. Lisa Beltz has helped address this need with the first edition of Emerging Infections: A Guide to Diseases, Causative Agents, and Surveillance. The text's stated objective is to fulfill the "… need for a college-level textbook in this field ..." and this goal was admirably attained.

The book comprises 30 chapters dealing primarily with selected microbial agents; 2 introductory chapters provide a useful basic summary of the scope of infectious diseases and emerging infections, as well as insights into host–agent interactions. Chapters on emerging infections in the immunocompromised host and on bioterrorism are also included. Each chapter is subdivided into 10 logical subunits that address the key aspects of each emerging infection. An initial major concepts section provides a chapter highlight and a bulleted summary, and key terms are included at the end of each chapter. Such repetition, which might seem redundant, is particularly effective at the undergraduate level.

Review questions and topics for further discussion are also provided. Although no references are included in the text, a series of resources is provided at the end of each chapter. The author borrows heavily from the excellent image library of the Centers for Disease Control and Prevention and this greatly enhances the book. Beltz has worked to make the material accessible, and the text is written largely in a colloquial and easy-to-understand fashion.

As would be expected in a first edition text covering such a broad topic, there are inevitable omissions and errata. It would have been useful to have expanded discussion, or perhaps added chapters, on zoonotic agents and their role in emerging infectious diseases, as well as additional information on the general principles of disease surveillance, and prevention and control approaches. There are minor misprints, such as the misspelling of cayatenensis, the use of syncytia (instead of syncytial), *Amoeba* (rather than *Entamoeba*) and humeral (instead of humoral). One notable omission is the absence of discussion of emerging helminthic diseases, such as angiostrongyliasis, cysticercosis, alveolar echinococcosis, and baylisascariasis. In addition, in an otherwise solid chapter on malaria, conspicuously absent was any discussion of the emergence of *Plasmodium knowlesi* in Southeast Asia. One could also argue for inclusion of different microbial agents than some of those presented, but I suspect that consensus on any such list would be unattainable.

Despite these drawbacks, Beltz has performed a fine job in producing a useful textbook on emerging infectious diseases that will work well in an upper-level undergraduate setting, as well as in courses for allied health professionals. Although this book might also be useful in graduate programs, supplementing it with journal articles and other sources will be necessary.

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Gustave Doré (1832–1883) Les Mendiants de Burgos (detail). 1875. 64 x 119 cm, 25.2 x 46.85 inches. Oil on canvas. Courtesy Galerie Michel Descours, Lyon, France

A Fragile Dignity Despite Their Rags and Tatters

Byron Breedlove

Gustave Doré, born in Strasbourg, France, in 1832, was one of the most prodigious artists of the 19th century. According to Dan Malan, one of his biographers, his nearly 10,000 engravings and 3,000 book editions made him the most prolific and popular illustrator of all time. Doré also produced around 400 oil paintings as well as watercolor landscapes, mixed media sketches, and sculptures. According to another Doré biographer, "The speed with which he drew was legendary, and his output was as noteworthy for its quantity as for its quality."

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Vincent van Gogh referred to Doré as an "Artist of the People" because Doré directed his work to the masses through his popular literary folios. He worked as a caricaturist and professional illustrator when he was just 15 years old. Doré illustrated texts by Dante, Rabelais, Perrault, Cervantes, Milton, Shakespeare, Hugo, Balzac, and Poe, plus the English Bible. The publication of his engravings in Dante's Inferno in 1861 brought him international fame.

Doré contributed 180 engravings to the book London: A Pilgrimage (1872), written by Blanchard Jerrold. Though commercially successful, the book spurred criticism as Doré included stark images of the poverty and despair he encountered during his time in London. The Westminster Review noted, for example, that "Doré gives us sketches in

which the commonest, the vulgarest external features are set down."

Throughout his career, Doré was sensitive to poverty, social misery, and human suffering. He traveled widely and sketched beggars, acrobats, and performers, often on the spot. He painted *Les Mendiants de Burgos* (or *Beggars of Burgos*), this month's cover image, in 1875.

Here a group of Castilian beggars cluster before a whitewashed wall as though they have assembled for a portrait. Most are dressed in tattered blankets, threadbare shawls, and disheveled clothing, though some wear colorful sashes and hats. A young mother, perhaps a recent widow, sits alone holding her infant. A crippled man lies in a small wooden wagon, his hands wrapped in leather or rags. Near the center, a tall man leans on walking sticks; one family with two small children stands to his left, while a couple sprawl on the sidewalk with an infant and a dog. Others beggars seen at the edges of the painting include a woman with a tambourine and several men who may have once been soldiers or tradesmen fallen on hard times. Whatever their stories, Doré paints these beggars, bound by poverty and lassitude, as still having a fragile dignity.

Now, as then, the poorest people, such as those depicted in Doré's *Les Mendiants de Burgos*, often live in the worst environments, are crowded together, lack adequate shelter, do not have clean water or sanitation, and suffer malnutrition—ideal circumstances for infectious disease transmission.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Legionnaires' Disease Incidence and Risk Factors, New York, New York, USA, 2002–2011

Streptococcus pneumoniae Serotype 8 Multidrug-Resistant Recombinant Clone Sweden-ST63, Spain

Respiratory Viruses and Bacteria among Pilgrims during the 2013 Hajj

Novel *Chlamydia trachomatis* Strains in Heterosexual Sex Partners, Indianapolis, Indiana, USA

Hepatitis E Virus Infections in Blood Donors, France

ESBL-producing *Salmonella enterica* Serovar Typhi in Traveler Returning from Guatemala, Spain

Human Influenza A(H7N9) Virus Infection Associated with Poultry Farm, Northeastern China

Raw Pig Blood Consumption and Potential Risk for *Streptococcus suis* Infection, Vietnam

Detection of Rare G3P[19] Group A Rotavirus in Human Patient, Italy

Severe Fever with Thrombocytopenia Syndrome Virus, South Korea, 2013

Mycobacterium tuberculosis Beijing Genotype Resistance to Transient Rifampin Exposure

Resolution Threshold of Current Molecular Epidemiology of Diphtheria

Zika Virus, French Polynesia, South Pacific, 2013

Antimicrobial Drug–Resistant Bacteria Isolated from Syrian War–Injured Patients, August 2011–March 2013

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

Upcoming Infectious Disease Activities

2014

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC, USA
http://www.icaac.org

October 8–12, 2014
ID Week 2014
Philadelphia, PA, USA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 2–6, 2014
ASTMH
American Society of Tropical Medicine and Hygiene
63rd Annual Meeting
New Orleans, LA, USA
http://www.astmh.org/Home.htm

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA, USA
http://www.apha.org/meetings/

November 30–December 4, 2014
ASLM2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

2015

March 8–11, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

2016

March 2–5, 2016
International Society for Infectious Diseases
17th International Congress on Infectious Diseases
Hyderabad, India
http://www.isid.org/icid/

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

Rapidly Growing Mycobacteria Associated with Laparoscopic Gastric Banding, Australia, 2005–2011

CME Questions

1. Your patient is a 50-year-old obese woman with fever and abdominal pain after undergoing laparoscopic gastric banding. According to the case series by Wright and colleagues, which of the following statements about the mode and characteristics of transmission of mycobacterial infections associated with laparoscopic gastric banding is correct?

A. Most cases were the result of *Mycobacterium abscessus*
B. The port was thought to be the primary site of infection in 17 of the 18 cases
C. Of the 18 patients, 16 were diagnosed with infection within the first 3 months after insertion
D. A total of 3 of these patients presented within 4 weeks of insertion with evidence of associated microperforation or erosion of the gastric lumen around the band site endoscopically or intraoperatively

2. According to the case series by Wright and colleagues, which of the following statements about clinical features and complications of mycobacterial infections associated with laparoscopic gastric banding is correct?

A. The most common symptoms associated with port infection were acute onset of fever, abdominal pain, nausea, and vomiting
B. A total of 10 patients initially appeared to have primary port site infection; 3 had concurrent port site and abdominal symptoms, and 5 patients presented with abdominal symptoms alone
C. Granulomatous peritonitis occurred in 2 patients with *M. fortuitum* confirmed on peritoneal biopsy
D. Chronic ulceration at the port site was not reported

3. According to the case series by Wright and colleagues, which of the following statements about management of mycobacterial infections associated with laparoscopic gastric banding would most likely be correct?

A. Empiric antibiotic therapy with a single agent is recommended
B. Device removal is usually not required
C. Current guidelines from the American Thoracic Society and the Infectious Diseases Society of America recommend combination therapy based on susceptibility testing
D. Macrolide resistance has not been reported in *M. fortuitum* and *M. abscessus*

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Risk Factors for Human Lice and Bartonellosis among the Homeless, San Francisco, California, USA

CME Questions

1. You are seeing a 46-year-old homeless man in the emergency department who complains of intermittent fever and severe headache for several days. The patient is disheveled and dirty, and there is evidence of body lice infection on his trunk. You are concerned that the patient might have trench fever. What should you consider regarding this diagnosis?

A. Conjunctival congestion and shin pain are common in trench fever
B. Mosquitoes, rats, and lice are known vectors of *Bartonella quintana*
C. *B. quintana* is usually excreted completely from the infected body louse in 1 to 2 days
D. Clinical infection is usually immediately apparent after exposure to *B. quintana*

2. The patient also has head lice. Which of the following statements regarding characteristics of homeless individuals with head lice in the current study is most accurate?

A. All participants had previously been infested with head lice
B. Most participants with head lice were African American
C. Nearly all participants with head lice slept in homeless shelters
D. Most participants with head lice exchanged clothing

3. Which of the following variables was a significant risk factor for body lice infestation among participants in the current study?

A. Female gender
B. White race
C. Sleeping outside
D. Exchanging clothing

4. According to the results of the current study, what should you consider regarding the potential for infection with *B. quintana* and trench fever in this patient?

A. The majority of body lice pools tested positive for *B. quintana*
B. The rate of positive testing results for *B. quintana* was higher in head lice vs body lice
C. All participants with concomitant head and body lice infestations tested positive for *B. quintana* at both anatomic sites
D. The pooled prevalence of *B. quintana* for head and body lice was 48%

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