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Fungal Infections

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On the Cover

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Alexandre Hogue, 1898–1994

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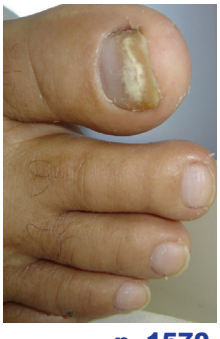

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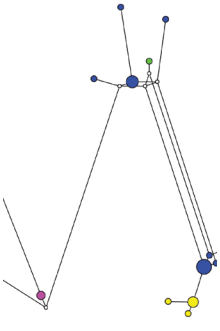

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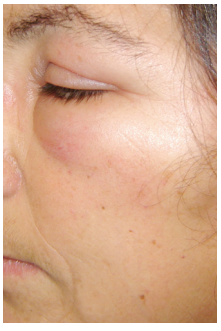

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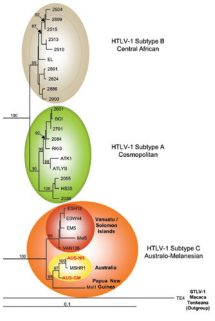

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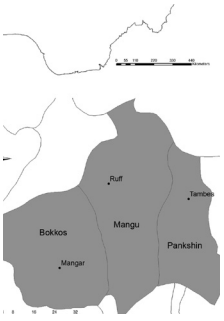

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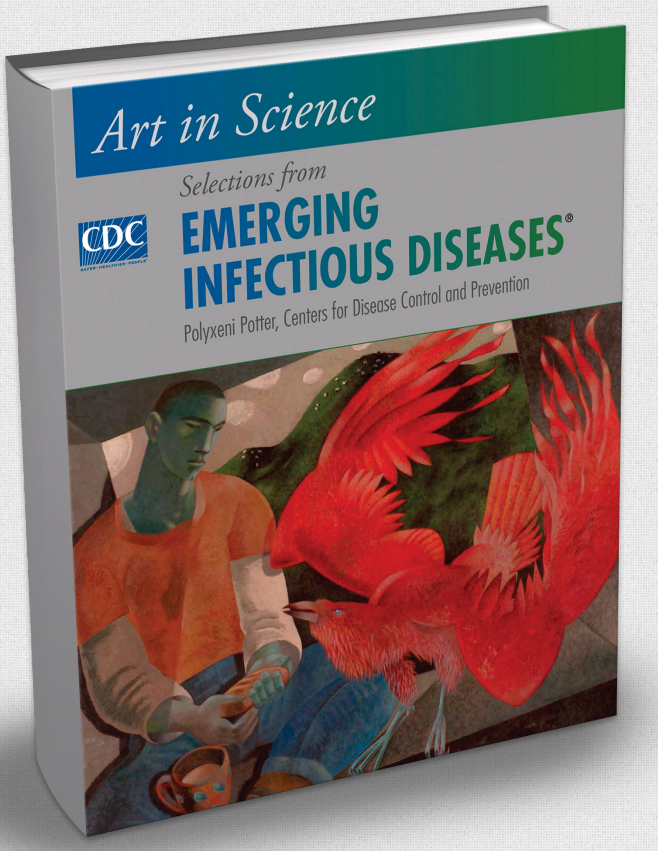

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Chagas Disease and Breast-feeding

Francesca F. Norman and Rogelio López-Vélez

Chagas disease (infection by the protozoan *Trypanosoma cruzi*) is a major parasitic disease of the Americas and one of the main neglected tropical diseases. Although various routes of transmission are recognized, the risk for transmission of the infection through breast-feeding has not clearly been established. We reviewed the literature on transmission of *T. cruzi* through breast-feeding to provide breast-feeding mothers with Chagas disease with medical guidance. Although data from animal studies and human studies are scarce, we do not recommend that mothers with Chagas disease discontinue breast-feeding, unless they are experiencing the acute phase of the disease, reactivated disease resulting from immunosuppression, or bleeding nipples. In these cases, thermal treatment of milk before feeding the infant may be considered.

Chagas disease (infection by the protozoan *Trypanosoma cruzi*) is a major parasitic disease in the Americas and one of the main neglected tropical diseases. *T. cruzi* is transmitted to humans primarily through the infected feces of triatomine bugs that contain metacyclic trypomastigotes (vectorial transmission). Other modes of transmission include transmission through infected blood and organs, vertical transmission, oral transmission (through consumption of contaminated food or drink), and transmission through laboratory accidents. The estimated number of infections in North, South, and Central America fell from ≈20 million in 1981 to ≈8–10 million in 2005; the reduction is mainly attributed to vector control campaigns and screening of blood donations in disease-endemic areas (*1*). However, the increase in mobile populations has led to the emergence of Chagas disease in non–disease-endemic areas where vectors are noncompetent and where the infection may be transmitted by other routes. A considerable proportion of migrants from Latin America are women, which turns the focus to preventing possible transmission from infected mother to child. The risk for congenital transmission from an infected mother may range from 0.13% to 17%, and the likelihood of congenital infection appears to correlate with maternal parasite density (*2*,*3*). Screening is recommended during pregnancy to detect mothers who may transmit *T. cruzi* to the fetus, even though no specific means of preventing congenital infection during pregnancy are available (*4*). The possibility of transmission through breast-feeding may be particularly relevant because, if breast-feeding is a route for transmission after delivery, such transmission may be preventable. Although some early publications identify breast-feeding as a possible route for *T. cruzi* infection, such data are scarce, and few animal studies exist (*5*–*8*).

Apart from other benefits, exclusive breast-feeding is the ideal way to provide nutrition during the first 6 months of life (*9*), and interruption of breast-feeding in resource-poor settings does not seem feasible unless the risks clearly outweigh possible benefits. If transmission occurs, treatment of acute infections is more successful in infants and children, and the treatment is also better tolerated (i.e., causes fewer side effects) in infants and children (*10,11*). The risk for transmission of infection through breast-feeding and whether any means are available to minimize this risk should be determined. The objective of this study was, therefore, to review the literature on transmission of *T. cruzi* through breast-feeding to adequately inform breast-feeding mothers with Chagas disease.

Search Strategy and Selection Criteria

We searched the literature in PubMed (www.ncbi.nlm.nih.gov/pubmed), EMBASE (www.elsevier.com/online-tools/embase), and Google Scholar (http://scholar.google.com/) for all published articles until January 2013, using the terms "breast-feeding" and "Chagas," "breastfeeding" and "*Trypanosoma cruzi*," "milk" and "Chagas," and "milk" and "*Trypanosoma cruzi*." No language, age, or sex restrictions were used. Both animal and human studies were considered. Direct access to some of the older publications was not possible, and information about results and conclusions was obtained from other articles.

Animal Studies

As early as 1913, Nattan-Larrier reported finding *T. cruzi* in the milk of laboratory animals (*12*). Disko and Krampitz found *T. cruzi* trypomastigotes in the milk of experimentally infected mice, which was not attributable to contamination of milk with blood. However, they did not subsequently demonstrate infection of suckling mice (*13*). In 1972, Miles examined the milk of mice in the acute phase of the disease and detected *T. cruzi* trypomastigotes

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and antibodies against *T. cruzi,* but concluded that, experimentally, even in the acute phase of the disease, transmission through breast-feeding was rare (*14*). In another study, Campos et al. found that nursing mice born to lactating females in the acute phase of the infection were not infected (*15*). Another study on vertical transmission of *T. cruzi* among chronically infected laboratory rats did not demonstrate transmission through breast-feeding, and histopathologic examination of the mammary glands of the infected rats did not reveal the parasites (*16*). More recently, Martins et al. inoculated 15 female mice with Y strain *T. cruzi* trypomastigote forms a few hours after they gave birth, and breast-feeding of offspring was continued. Despite confirmed infection in female mice, no infection through breast-feeding was documented in the 142 offspring examined (examination of fresh blood by microscopy) (*17*). Other authors have not been able to confirm experimental transmission through breast-feeding during the acute phase of the disease (*18*–*21*), and researchers have hypothesized that maternal antibodies transferred with the milk may play a protective role (*14*,*22*).

Studies that examined pasteurization and microwave treatment of human milk to prevent transmission of Chagas disease have demonstrated transmission when human milk contaminated with Y strain trypomastigotes was orally administered to mice. However, such transmission occurred under experimental conditions, not natural breast-feeding, and the milk was artificially contaminated. In 1 experiment, batches of laboratory mice were orally or intraperitoneally inoculated with human milk that was contaminated with *T. cruzi* trypomastigotes and pasteurized, contaminated with *T. cruzi* and not pasteurized, or not contaminated with *T. cruzi*. Only mice that were given contaminated nonpasteurized milk (orally or intraperitoneally) were infected (detected by examination of peripheral blood) (*23*). In a similar study, mice that received either infected or noninfected microwave-treated milk (63°C, domestic microwave oven 2,450 MHz, 700 W), orally or intraperitoneally, were not infected (negative parasitologic and serologic results), whereas mice that received contaminated milk that had not been microwaved acquired the infection (*24*).

Studies involving metacyclic trypomastigotes from triatomine feces have found that contaminated milk seems to be an adequate medium (when compared with other types of food and water) for oral transmission of *T. cruzi* to mice, and this fact has been attributed to its moisture and nutrient content (*25*). In certain animal experiments, infection through the oral route (not breast-feeding) has been established by using blood trypomastigotes, and the success of this mode of transmission appeared dependent on the size of the inoculum used (*26*,*27*). Transmission of *T cruzi* by breast-feeding is possible in animal models, but oral infections may be difficult to establish unless metacyclic forms of the parasite are involved.

Studies in Humans

Two reports from 1936 and 1983 describe finding trypomastigotes in the milk of mothers in the acute phase of Chagas disease. Mazza et al. (*5*) reported transmission through lactation; in the other study, the newborn was not breast-fed (*28*). However, another publication later indicated that in the study by Mazza et al., the collected milk was contaminated by blood (*29*). Medina-Lopes reported 2 cases of acute Chagas disease in infants that had been acquired through breast-feeding from mothers in the chronic phase. In 1 of these cases, no parasites were found in the milk, and the source of the infection was postulated to be infected blood because the mother had bleeding nipples. In the other case, congenital, vector-borne, and transfusion-associated transmission were excluded, and transmission was suspected to have occurred through breast-feeding (*6*,*7*).

In 1988, Bittencourt et al. were unable to demonstrate transmission of infection through breast-feeding. Samples of milk/colostrum from 78 mothers in the chronic phase of the disease were studied (5 mothers had parasitemia at the time of milk collection). Results of the following were negative: the parasitologic study of all sample-inoculated mice and the serologic tests of serum samples from 97 breast-fed children born free of infection (*22*).

In 1990, Shikanai-Yasuda et al. published a series of reports of cases of acute Chagas disease, and breast-feeding was considered a possible route of transmission for 2 patients (*30*). Amato Neto et al. (*31*) could not document the presence of *T. cruzi* in the colostrum and breast milk of 40 women with Chagas disease through direct observation, culture, and inoculation. The possibility of using a larger study population and/or more efficient diagnostic techniques was highlighted (*31*).

Other studies that examined maternal transmission of *T. cruzi* included breast-feeding as a possible contributing factor, but once again, this mode of transmission could not be demonstrated. In 2004, Rassi et al. found 2 cases of maternal transmission of infection in 2 patients who had also been breast-fed, but this mechanism could not be established, and the infections may have resulted from vertical transmission (*32*).

The data on transmission of *T. cruzi* through breast-feeding in humans are scarce; reports are not recent and the findings have several limitations. Additional data on methods used to diagnose infection and exclude other forms of transmission in these studies are summarized in the Table.

Discussion

In mice, oral transmission of *T. cruzi* infection through human milk contaminated with trypomastigotes is possible,

although natural transmission through breast-feeding has not been clearly demonstrated in recent studies. Infection in laboratory mice acquired after oral or intraperitoneal administration of human milk contaminated with *T. cruzi* trypomastigotes may be prevented by thermal treatment (pasteurization or microwave treatment at 63°C) of milk, which can inactivate the trypomastigotes. Such treatment of milk may therefore be an option for lactating mothers who have the acute form of the infection or bleeding nipples.

In humans, the oral route is an efficient mode of transmission for *T. cruzi*, as has been demonstrated in recent outbreaks of infection acquired through contaminated food and fruit juices (*33*–*35*). Ingestion of food or drink contaminated with vector feces (containing metacyclic trypomastigotes) or crushed insects is the most likely mode of oral infection (*36*). The concentration of the ingested inoculum may result in the different clinical manifestations and variations in attack rates reported in outbreaks (*34*).

Milk appears to be an adequate medium for transmission of *T. cruzi* because of its moisture and nutrient content and, in the case of human milk, body temperature would also be favorable (the parasite can be destroyed in dry vector feces and by high temperatures) (*23*,*24*,*36*). Despite this, except for some dated cases, in humans, transmission through breast-feeding has not been reported.

A further consideration to account for the relative lack of infections transmitted through breast-feeding is that, if they occur at all, blood-form trypomastigotes would be expected to occur in human milk and not metacyclic trypomastigotes, which are found in the vector. Blood,

Table. Summary of human studies on transmission of *Trypanosoma cruzi* through breast-feeding*

Reference	Phase of infection, mother	Methods and findings	Comments
Mazza et al, 1936 (*5*)	Acute	No trypomastigotes detected by direct methods in newborn at d 10. Parasites detected at 3 mo when acute Chagas disease diagnosed in newborn and concomitantly found by direct methods in mothers´ milk	Congenital transmission not adequately ruled out. Detection of parasites in milk may have been due to contamination with maternal blood containing trypomastigotes from a bleeding nipple (*29*).
Medina-Lopes and Macedo, 1983 (*28*)	Acute	Wk 5 of maternal acute infection (before delivery): direct examination of colostrum: negative; intraperitoneal inoculation of mice with colostrum and xenodiagnosis: positive. Maternal milk (after delivery) intraperitoneally inoculated into mice and xenodiagnosis: positive	Demonstrated *T.cruzi* in colostrum and milk. Contamination from blood not mentioned but unlikely as newborn did not breast-feed (assuming intact nipples during sample extraction). Congenital infection ruled out by using 4 different (unspecified) diagnostic methods
Medina-Lopes, 1983 (*6*)	Chronic	Congenital transmission excluded by unspecified methods. Vectorial transmission excluded. Acute infection diagnosed in 2-mo-old newborn by unspecified methods.	Mother had nipple bleeding: infected blood in milk may have been the source of the infection. Milk direct exam was negative.
Medina-Lopes, 1988 (*7*)	Chronic	Infection excluded at birth by modified Strout and xenodiagnoses by using cord blood. At 6 mo IFAT and IHA: positive. At 7 mo modified Strout: positive. Transfusional and vectorial transmission excluded (housing inspected for vector)	Mother had positive xenodiagnosis at time of delivery. Mother had nipple fissures 1 wk postpartum; unclear whether breast-feeding occurred at time. No examination of maternal milk performed. Older sibling not infected even though also breast-fed at the same time.
Bittencourt et al., 1988 (*22*)	Chronic	Milk/colostrum from 78 mothers (101 samples) studied by direct examination and inoculation of mice. Mice tested by direct blood examination, xenodiagnosis, and IFAT for *T. cruzi* antibodies. No mice infected. Transmission in 97 breast-fed children excluded at birth by direct blood examination-microhematocrit and xenodiagnosis and by serology (IFAT) at 6–24 mo.	5 mothers had positive blood xenodiagnosis at time of milk collection. Mothers recommended to avoid breast-feeding if nipple bleeding. Average time of breast-feeding was 7 mo; unclear whether any infant tested by serology before ending breast-feeding-
Shikanai-Yasuda et al., 1990 (*30*)	Chronic	Acute Chagas disease diagnosed at 3.5 and 9.5 mo in 2 infants, respectively, by direct examination of peripheral blood.	Vectorial transmission unlikely (urban area). Congenital transmission not investigated. Mother of the 9.5-mo-old infant had nipple fissures and bleeding during breast-feeding.
Amato Neto et al., 1992 (*31*)	Chronic	Search for *T. cruzi* in colostrum and breast milk of 40 chronically infected women through direct observation, culture, and inoculation	No evidence of *T. cruzi* in samples
Rassi et al., 2004 (*32*)	Chronic	Identified 2 cases of infection in children 2- and 5-y-old, respectively, detected by at least 2 positive serologic tests (complement fixation, IHA, ELISA, and IFAT)	Transfusional and vectorial transmission excluded. Probable congenital infections but both patients also breast-fed and contribution of this factor could not be ruled out

*IFAT, indirect immunofluorescence antibody test; IHA, indirect hemagglutination antibody test.

metacyclic, and culture trypomastigotes are similar in form and structure, but the different forms may have different surface glycoproteins, which are used for cell invasion (*17*,*37*). Metacyclic trypomastigotes may have specialized surface molecules (such as gp82, a member of the gp85/trans-sialidase superfamily, or pepsin-susceptible gp90 isoforms, which make parasites highly invasive against target cells after contact with gastric juice) with functions for mucosal invasion that are not present in blood-form trypomastigotes. The latter, in turn, express other molecules that facilitate dissemination within the host (*38*–*40*). Even though trypomastigote forms of the Y strain of *T. cruzi*, which expresses glycoprotein 82 in metacyclic forms and should have high infectivity, were used in some experiments, transmission through breast-feeding could not be demonstrated. Blood-form trypomastigotes may therefore not be as efficient as metacyclic trypomastigotes in establishing mucosal invasion of the gastrointestinal tract (partly because of their decreased capacity to migrate through the gastric mucin layer), even though oral infections with blood trypomastigotes have been demonstrated experimentally with the possibility of infection depending on amount of inoculum. Peptic digestion may also destroy some of the infective forms, mainly blood trypomastigotes (*40*).

On the basis of the data examined, oral infection through breast-feeding does not appear to be an efficient mode of transmission for *T. cruzi*. In humans, milk contamination by *T. cruzi* has been described only in isolated cases. In the acute phase, patent parasitemia occurs, but *T. cruzi* has been demonstrated in breast milk only in 1 case (transmission did not occur because the newborn was not breast-fed) (*27*). In the chronic phase, parasitemia is usually low grade and tends to fluctuate. A couple of cases of acute infection in breast-feeding infants of mothers in the chronic phase have been reported, but transmission may have resulted from the ingestion of blood from bleeding nipples (*6*,*7*). In older studies, it is not always clear how accurately vertical and other modes of transmission had been excluded with the diagnostic tools available at the time, and in other cases, breast-feeding is contemplated only as a possible route of infection. Many of the studies reported were performed some years ago, and current novel techniques, such as PCR, may aid in detecting low parasite loads. On the other hand, new techniques may even yield equivocal results (e.g., because of the presence of infected blood but not true contamination of milk by *T. cruzi*) if contamination with infected blood were below the level of detection found with other methods. In humans, transmission of *T. cruzi* through maternal milk (not contaminated with infected blood) may not be considered clearly proven.

As previously postulated in laboratory studies, in humans, a combination of several factors likely account for the low efficiency of breast-feeding as a possible mode of transmission of *T. cruzi* infection. These include the low number of trypomastigotes ingested by the lactating infant, the biologic forms and strains of *T. cruzi* involved, the passage of protective maternal antibodies, and the destruction of some parasites by gastric juice.

A limitation of this review was that some of the articles dated back several decades and were not readily available. Some information about these studies was obtained from other articles.

Published studies on the transmission of *T. cruzi* through breast-feeding are scarce. After reviewing the various studies, and given the demonstrated benefits of breast-feeding (especially in lower income disease-endemic countries where a safe and inexpensive alternative may not be available), we conclude that discontinuing or interrupting breast-feeding by mothers with chronic Chagas disease is not recommended. If the mother has fissures or bleeding nipples, temporary discontinuation of breast-feeding may be recommended, although thermal treatment (pasteurization or microwaving) of expressed milk before feeding the infant may be a safe alternative. When mothers are in the acute phase of the disease (an infrequent situation that will occur almost exclusively in disease-endemic countries) or have reactivated disease (resulting from immunosupression, in which circulating parasitemia level may be high), breast-feeding may pose a risk for the infant. The benefits of using thermally treated expressed milk should be considered on an individual basis. To avoid any possibility of transmission, human milk banks should exclude mothers with Chagas disease as donors. As other modes of transmission of Chagas disease come under control (vector-borne transmission and transmission through infected blood/organs), further studies are needed on the possibility of transmission through breast-feeding, as this could occur even in countries where the disease is nonendemic.

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etymologia

Chagas Disease

[shä-gəs] Disease

Prevalent among persons who have lived in Mexico, Central America, and South America, Chagas disease can cause chronic and potentially severe cardiac and gastrointestinal disease decades after infection. The disease is named for Carlos Chagas, a Brazilian scientist who discovered a new species of *Trypanosoma* in the intestines of triatomine insects (called *barbeiro* or barber because they often bite the face). In 1908, Chagas named the new species *T. cruzi* after his mentor, Oswaldo Cruz. The next year, he identified the parasite in the blood of an ill 2-year-old girl named Bérénice, in what became the first description of this new human disease.

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Increased Incidence of Invasive Fusariosis with Cutaneous Portal of Entry, Brazil

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Release date: September 18, 2013; Expiration date: September 18, 2014

Learning Objectives

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

- Distinguish the most common underlying diagnosis of patients with invasive fusariosis in the current study
- Analyze the clinical presentation of patients with invasive fusariosis
- Assess the laboratory diagnosis and management of invasive fusariosis
- Evaluate trends in the incidence of fusariosis.

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Invasive fusariosis (IF) is an infection with *Fusarium* spp. fungi that primarily affects patients with hematologic malignancies and hematopoietic cell transplant recipients. A cutaneous portal of entry is occasionally reported. We reviewed all cases of IF in Brazil during 2000–2010, divided into 2 periods: 2000–2005 (period 1) and 2006–2010 (period 2). We calculated incidence rates of IF and of superficial infections with *Fusarium* spp. fungi identified in patients at a dermatology outpatient unit. IF incidence for periods 1 and 2 was 0.86 cases versus 10.23 cases per 1,000 admissions (p<0.001), respectively; superficial fusarial infection incidence was 7.23 versus 16.26 positive cultures per 1,000 superficial cultures (p<0.001), respectively. Of 21 cases of IF, 14 showed a primary cutaneous portal of entry. Further studies are needed to identify reservoirs of these fungi in the community and to implement preventive measures for patients at risk.

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Invasive fusariosis (IF) is a mycosis caused by infection with *Fusarium* spp. fungi that affects primarily patients with hematologic malignancies and hematopoietic cell

transplant (HCT) recipients (*1,2*). In these severely immunosuppressed patients, IF is typically disseminated and involves pneumonia, metastatic skin lesions, and positive blood cultures (*3*). The usual portal of entry is the airways, and IF is thought to be acquired by the inhalation of aerosols of fusarial conidia. However, the skin at sites of tissue breakdown may be a portal of entry (*4*). In a review of 232 published cases of IF in immunosuppressed patients, primary skin lesions represented the likely portal of entry in 16 (11%) of 147 patients with disseminated disease (*5*).

In 2007, we observed an increase in the incidence of IF in our hospital in Brazil: 5 cases of disseminated IF and 2 cases of locally invasive disease were diagnosed in a 7-month period. In addition to this apparent increase in incidence, we observed that all patients had a primary skin lesion on the lower limbs. All case-patients were housed in 4 rooms of the hematology unit. Because of the increasing incidence and the cutaneous primary lesions, environmental sampling of air, water, and water-related structures of the hematology unit were conducted; we also performed molecular analysis of patient and environmental isolates. We describe the incidence, clinical presentation, and outcome of these cases and compare these results with cases of superficial *Fusarium* spp. infection among outpatient dermatologic patients at the same hospital.

Patients and Methods

The University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, is a tertiary-care teaching hospital with ≈450 beds and a hematology unit that has 5 double-bed rooms without high-efficiency particulate air filtration and 8 single-bed rooms equipped with filters and positive pressure. Cases of IF were identified during daily visits to the hematology ward, by review of a database of episodes of febrile neutropenia (active since 1986), and by review of the hospital's mycology laboratory and pathology registries. Typically, the diagnosis of IF is confirmed by blood culture and/or by direct exam, culture, and histopathology of metastatic skin lesions, when present (*3*).

Cases of superficial infections caused by *Fusarium* spp. were identified by reviewing the Mycology Laboratory database of cultures of dermatologic patients, a database that contains description of the type of lesion from which direct examination and culture were performed, as well as the results of direct exam and culture. These infections were diagnosed in outpatients who attended the hospital's dermatology clinic, and the diagnosis required the presence of a superficial lesion with positive culture of the lesion.

We reviewed the records of all patients in whom IF was diagnosed during 2000–2010, obtaining detailed information on demographics, underlying disease and treatment, comorbidities, presence of neutropenia, receipt of corticosteroids and other immunosuppressive agents, clinical manifestations of IF, diagnosis, treatment, and outcome. All patients had been hospitalized for the treatment of an underlying hematologic condition and had fusariosis develop in the context of immunosuppression caused by the underlying disease and its treatment. IF was defined as the isolation of *Fusarium* spp. from any sterile biologic material, such as blood or skin biopsy, or from respiratory secretions in patients with typical clinical signs, including fever and metastatic skin lesions (*1*). A cutaneous portal of entry was defined when the clinical manifestations (and the diagnosis) of IF were preceded by the occurrence of localized skin lesions (such as cellulitis at sites of onychomycosis and intertrigo) with positive culture for *Fusarium* spp. The cases of IF were classified as proven or probable, according to the modified criteria of the European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group and the National Institute of Allergy and Infectious Diseases Mycoses Study Group Consensus Group (*6*). Superficial infections caused by *Fusarium* spp. in immunocompetent patients from the dermatology clinic were defined when *Fusarium* spp. was recovered from a skin lesion (usually onychomycosis and intertrigo). No changes in the population at risk, standards of collection and processing of biologic material, and diagnostic capabilities in the mycology laboratory occurred during the study period.

For the purpose of estimating changes in the incidence of IF, we split the study period into 2 periods: 2000–2005 (period 1) and 2006–2010 (period 2). We calculated the incidence of IF for the 2 periods using total admissions in the hematology unit as denominator and expressing the rates as number of cases per 1,000 admissions. The incidence of superficial infections caused by *Fusarium* spp. was expressed as number of positive cultures per 1,000 superficial cultures processed. Incidence densities between different periods were compared by the χ^2 test using Epi Info software version 6.04d (Centers for Disease Control and Prevention, Atlanta, GA, USA). We considered p values <0.05 as statistically significant.

Results

During 2000–2010, a total of 21 cases of IF were diagnosed in patients in the hematology unit at the hospital (Table). Acute myeloid leukemia (AML) was the most frequent underlying disease (42.9%); 12 patients (57.1%) were HCT recipients (8 allogeneic, 4 autologous). Neutropenia (81.0%), receipt of corticosteroids (76.2%), and graft-versus-host disease (6 of 8 allogeneic HCT recipients) were the most frequent predisposing factors. The IF diagnosis was confirmed by blood culture alone in 7 cases, blood culture plus culture and histopathology of biopsy of a metastatic skin lesion in 4, culture and histopathology of skin biopsy in 7, culture of synovial fluid in 2, and culture

Table. Characteristics of 21 patients with invasive fusariosis in the hematology united at University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, 2000–2010*

Characteristic	Patients
Sex, M:F	16:5
Median age, y (range)	55 (9–71)
Underlying disease	
Acute myeloid leukemia	9 (42.9)
Multiple myeloma	4 (19.0)
Non-Hodgkin lymphoma	2 (9.5)
Acute lymphoid leukemia	2 (9.5)
Myelodysplasia	2 (9.5)
Aplastic anemia	1 (4.8)
Chronic myeloid leukemia	1 (4.8)
HCT recipients	12 (57.1)
Allogeneic	8 (38.1)
Autologous	4 (19.0)
Room with HEPA filter	14 (66.7)
Receipt of corticosteroids	16 (76.2)
Graft-versus-host disease, n = 8†	6 (75.0)
Neutropenia	17 (81.0)
Skin as portal of entry	17 (81.0)
Positive blood culture	11 (52.4)
Classification of fusariosis	
Proven	20 (95.2)
Probable	1 (4.8)
Primary treatment	
None	1 (4.8)
Voriconazole	7 (33.3)
Deoxycholate amphotericin B	10 (47.6)
Deoxycholate amphotericin B + voriconazole	3 (14.3)

*Values are no. (%) patients except as indicated. HCT, hematopoietic cell transplant; HEPA, high-efficiency particulate air.
†Allogeneic HCT recipients.

of sinus aspirate in 1. Among the 20 patients who received treatment, deoxycholate amphotericin B (10 patients) and voriconazole (7 patients) were the most frequent agents used as primary therapy. The overall 30-day and 60-day survival rates for the 21 patients were 33.3% and 28.6%, respectively.

A cutaneous portal of entry was identified in 14 (66.7%) of the 21 cases: onychomycosis with periungueal cellulitis in 6 cases, onychomycosis with interdigital intertrigo in 1 case, intertrigo with lymphangitis in 2 cases, intertrigo alone in 4 cases, and ulcer in 1 case (Figure 1, panels A–D). Some of the lesions of intertrigo evolved to necrosis and tissue loss (Figure 1, panel E). The median time from admission to recognition of a cutaneous portal of entry was 11 days (range –11 to 65).

Figure 2 shows the incidence of IF during the full study period. The incidence was 0.86 cases per 1,000 admissions in period 1 and 10.23 cases per 1,000 admissions in period 2 ($p<0.001$). The incidence of IF with a cutaneous portal of entry was 0.43 per 1,000 admissions in period 1 and 6.99 per 1,000 admissions in period 2 ($p<0.001$).

Figure 3 shows the incidence of superficial infection caused by *Fusarium* spp. in patients from the dermatology outpatient clinic. The incidence (positive cultures per 1,000 superficial cultures) was 7.23 in period 1 and 16.26 in period 2 ($p<0.001$).

Isolates from hematologic and dermatologic patients showed similar species distribution, with a predominance of *F. solani* species complex (FSSC) species 2 (69% and 74%, respectively). By contrast, most environmental isolates from the initial investigation were identified as *F. oxysporum* species complex, with very few isolates of FSSC species 2, suggesting that most of the clinical cases of IF we identified would not be traceable to a specific environmental source in the hospital ward (*7*).

Discussion

We observed an increase in the incidence of IF over time among patients in the hematology ward of our hospital; a cutaneous portal of entry was evident in most cases. We also observed an increase in the incidence of superficial infections caused by *Fusarium* spp. in outpatient nonhematologic patients at the same hospital during the same period.

The increase in the incidence of IF from period 1 (2000–2005) to period 2 (2006–2010) was >10-fold and showed a clear upward trend. Although IF is considered an emerging invasive fungal disease, affecting mostly patients with hematologic malignancies (*3*), its incidence is usually low. An epidemiologic study conducted in 18 hospitals in Italy reported 15 cases of IF among 11,802 patients with hematologic malignancies; patients with AML had the highest incidence (13 cases in 3,012 patients) (*8*). Another Italian study reported 3 cases among 1,249 allogeneic HCT recipients (*9*). In the United States, a large prospective study performed in 21 centers (1,194 allogeneic HCT recipients) reported only a few cases of infection caused by non–*Aspergillus* spp. molds, with a <0.3% 1-year cumulative incidence (*10*). By contrast, a prospective study conducted in 8 centers in Brazil during 2007–2009 reported 23 episodes of IF in 937 hematologic patients (2.4% overall incidence rate) (*11*); this study found a 1-year cumulative incidence of 5.2%, 3.8%, and 0.6% among allogeneic HCT recipients, AML patients, and autologous HCT recipients, respectively. No center effect was observed to account for this high incidence.

We also found a high incidence of a cutaneous portal of entry for IF, which is in sharp contrast with what had been previously reported. Similar to invasive aspergillosis, IF is thought to be acquired by inhalation of conidia from the air but occasionally has a cutaneous portal of entry (*4*). In a review of 259 published cases of IF, a cutaneous portal of entry was reported for only 11% of cases, and these were nearly all restricted to onychomycosis as the primary lesion (*5*). By contrast, 14 (66.7%) of the 21 IF cases in our study had a cutaneous portal of entry. Molecular typing of isolates recovered from sites of invasive disease (blood, synovial fluid) and from the lesions in the feet thought to be the portal of entry was performed for 4 cases and showed the same species for 3 (*7*).

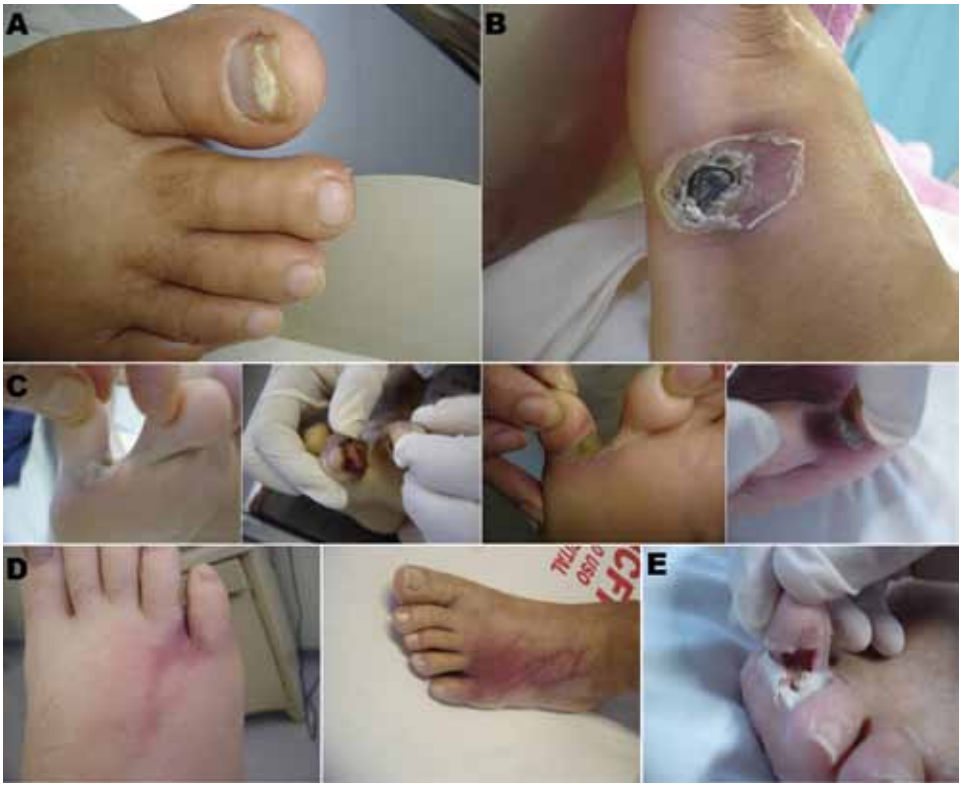

Figure 1. Primary skin lesions in patients with invasive fusariosis in the hematology unit at University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, 2000–2010. A) Onychomycosis; B) ulcer; C) intertrigus; D) intertrigus evolving to lymphangitis before dissemination. (First image in D is the same patient as the first image in C; second image in D is the same patient as the fourth image in C.) E) Necrosis in a lesion of intertrigus (evolution of the lesion shown in the third image in C).

Our results showed that interdigital intertrigo was as common as onychomycosis, occurring in 7 of the 14 cases with a cutaneous portal of entry. Onychomycosis is usually caused by *Candida* spp. and dermatophytes (*12*), but fusarial onychomycosis is a known clinical entity (*13*). Furthermore, recent studies have suggested that nondermatophyte fungi (including *Fusarium* spp.) are emerging as causes of onychomycosis (*14–16*). By contrast, interdigital intertrigo is rarely caused by *Fusarium* spp. (*17*).

The increased incidence of IF observed in 2007 at our hospital and the unique aspect of a cutaneous portal of entry in most cases raised the possibility that the patients could have acquired IF by contact with contaminated water in the hospital. Our hypothesis was that patients had been admitted with subtle skin breakdowns that became colonized by *Fusarium* spp. after contact with the hospital water, and local infection and dissemination subsequently developed. In support of this hypothesis were the findings of Anaissie et al., who reported that *Fusarium* spp. were recovered from 57% of water samples and 88% of water-related structures in a hospital in the United States; molecular studies of the isolates revealed a close relatedness between patient and

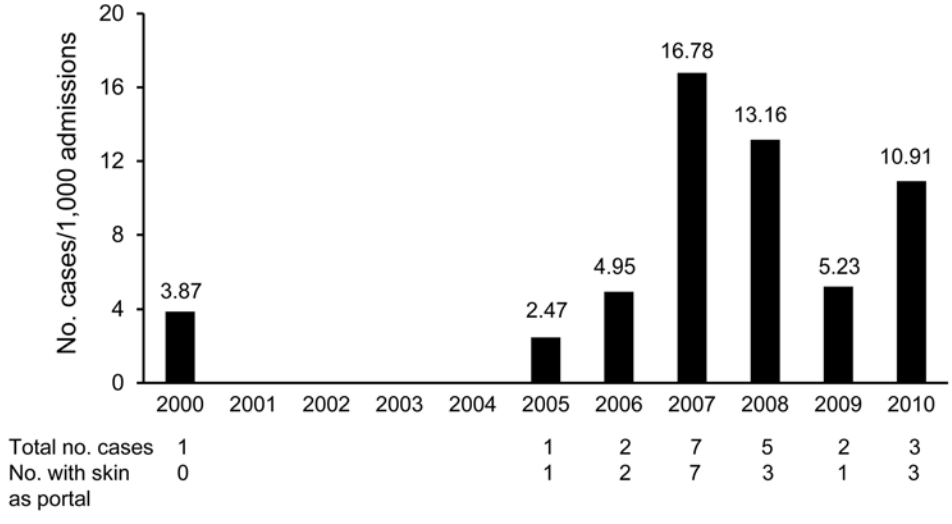

Figure 2. Incidence of invasive fusariosis among patients in the hematology unit at University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, 2000–2010.

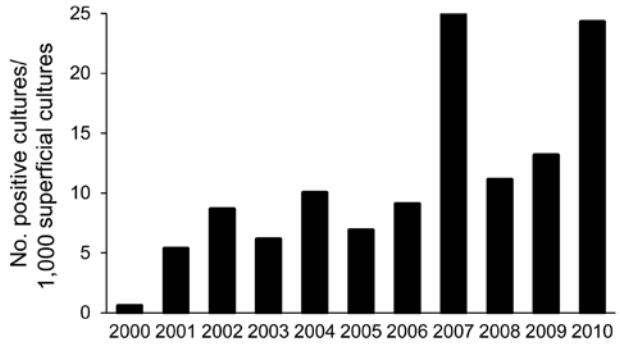

Figure 3. Incidence of superficial infections caused by *Fusarium* spp. among outpatients at the dermatology clinic of University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, 2000–2010.

environmental isolates (*18*). However, our environmental investigation showed that, although *Fusarium* spp. were present in the hospital water system, most isolates from patients belonged to the FSSC 2, whereas environmental isolates belonged to the *F. oxysporum* species complex (*7*). These results suggest that the infection did not have a nosocomial origin.

Concomitant to the increase in IF, we recorded an increase in the growth of *Fusarium* spp. from superficial infections in outpatients, from 7.23 positive cultures for period 1 to 16.26 positive cultures per 1,000 superficial cultures for period 2. Considering this apparent emergence of fusarial superficial infections in the community, the immunocompromised patients served as sentinels for the detection of this problem (*19*).

A limitation of our study is the denominator used to calculate the incidence. Because infection was acquired in the community, the appropriate denominator would be population based. However, because obtaining such a denominator would be difficult, we used a hospital-based denominator to approximate the incidence.

Our findings may have implications for future research, in particular, determining the environmental reservoirs of *Fusarium* spp. in the community that promoted the emergence of superficial fusariosis in immunocompetent patients. *Fusarium* spp. are widely found in the environment and are pathogens of various plants, including tomatoes, soybeans, and various grains (*20*). One possibility for an increase in *Fusarium* spp. in the environment is agricultural activities. For example, the Cerrado area is a large (≈2 million m^2) territory that encompasses 10 states of Brazil. During the past 15–20 years, the area underwent a great deal of change in its composition, with a massive replacement of the native vegetation with monoculture, typically soybeans and pasture (*21*). A study evaluating the fungal diversity of the region found a great loss of fungal richness and diversity in the soybean plantation and pasture areas compared with native vegetation, with a concentration of ascomycetes (*22*). Other questions that require future research include assessment of the frequency and clinical significance of baseline skin colonization with *Fusarium* spp. in immunosuppressed patients and evaluation of preventive measures to reduce the incidence of this devastating disease.

In conclusion, we observed an increase in the incidence of IF in our hematology ward, with a cutaneous portal of entry, and of superficial fusariosis in immunocompetent outpatients. Future studies are needed to identify reservoirs of *Fusarium* spp. in the community, as well as preventive measures for patients at high risk for IF.

Dr Nucci is associate professor in the Department of Internal Medicine and head of the Mycology Laboratory of the University Hospital, Federal University of Rio de Janeiro, Brazil. His primary research interests are supportive care in hematologic patients, infections in hematologic patients and hematopoietic cell transplant recipients, and nosocomial fungal infections.

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Genetic Recombination and *Cryptosporidium hominis* Virulent Subtype IbA10G2

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Little is known about the emergence and spread of virulent subtypes of *Cryptosporidium hominis*, the predominant species responsible for human cryptosporidiosis. We conducted sequence analyses of 32 genetic loci of 53 *C. hominis* specimens isolated from a longitudinally followed cohort of children living in a small community. We identified by linkage disequilibrium and recombination analyses only limited genetic recombination, which occurred exclusively within the 60-kDa glycoprotein gene subtype IbA10G2, a predominant subtype for outbreaks in industrialized nations and a virulent subtype in the study community. Intensive transmission of virulent subtype IbA10G2 in the study area might have resulted in genetic recombination with other subtypes. Moreover, we identified selection for IbA10G2 at a 129-kb region around the 60-kDa glycoprotein gene in chromosome 6. These findings improve our understanding of the origin and evolution of *C. hominis* subtypes and the spread of virulent subtypes.

Cryptosporidium spp. are emerging pathogens of humans and a variety of vertebrates, and cause severe diarrhea in immunocompetent and immunocompromised persons (*1*). *Cryptosporidium hominis* is responsible for >70% of human infections in most areas, especially North America and developing countries (*2*). *C. hominis* is primarily transmitted anthroponotically, has several transmission routes, and causes numerous waterborne outbreaks of diarrheal illness each year in the United States and other industrialized nations.

Among several *C. hominis* subtype groups (e.g., Ia, Ib, Id, Ie, If, Ig) identified by sequence analysis of the 60-kDa glycoprotein (gp60) gene, the Ib subtype is the major subtype responsible for waterborne and foodborne outbreaks of cryptosporidiosis in many countries. Subtype IbA10G2 has been found in ≈50% of *C. hominis*–associated outbreaks in the United States, including the massive outbreak in Milwaukee, Wisconsin, USA, in 1993 (*2*,*3*). It is the only subtype identified in cryptosporidiosis outbreaks by *C. hominis* in countries in Europe and in Australia (*4*–*11*). In a longitudinal birth-cohort study of cryptosporidiosis in a periubran shantytown in Lima, Peru, IbA10G2 was more virulent than other *C. hominis* subtypes (*12*). Genetic determinants for virulence of *Cryptosporidium* spp. and reasons for emergence of virulent subtypes are poorly understood because of availability of only limited genomic sequence data and lack of robust cultivation systems and genetic manipulation tools (*13*).

We conducted a comparative population genetic analysis of virulent *C. hominis* subtype IbA10G2 in children living in a periurban community in Lima, Peru, by multilocus sequence typing (MLST) of 32 genetic markers. Data obtained should be useful in understanding emergence and spread of virulent *C. hominis* subtypes.

Materials and Methods

Specimens, Species, and Subtype Determination

Fecal specimens microscopically positive for *Cryptosporidium* spp. were collected during a longitudinal cohort study of enteric diseases in children living in a periurban shantytown in Lima, Peru, during 2004–2006. The field study was conducted in the same community used in a previous longitudinal cohort study of enteric pathogens and had a similar study design (*12*), except that children enrolled in this study were older (mean age 3.43 years vs. 14 days). The study was approved by the institutional review boards of Johns Hopkins University and the Centers for Diseases Control and Prevention.

DNA was extracted from 200 μL of microscopy-positive specimens by using the FastDNA SPIN Kit for

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Soil (MP Biomedicals, Irvine, CA, USA). *Cryptosporidium* spp. in specimens were genetically characterized at the species level by PCR–restriction fragment length polymorphism analysis of the small subunit rRNA gene (*14*) and at the subtype level by DNA sequence analysis of the gp60 gene (*15*). A total of 53 *C. hominis*–positive specimens (1 specimen/child) belonging to 4 gp60 subtype groups were selected for MLST analysis: Ia (9 specimens of IaA13R8 and 1 specimen of IaA13R7), Ib (26 specimens of IbA10G2), Id (6 specimens of IdA10 and 5 specimens of IdA20), and Ie (6 specimens of IeA11G3T3). No specimens used in the study had mixed *C. hominis* subtypes.

MLST Markers

Among the 8 chromosomes of *Cryptosporidium* spp., chromosome 6 was fully sequenced for 2 *C. parvum* specimens and 1 *C. hominis* specimen, which facilitated search for additional microsatellite and minisatellite markers for population genetics analysis. The 8 polymorphic markers on chromosome 6 (gp60, CP47, CP56, MSC6–5, MSC6–7, TSP8, Mucin1, and DZ-HRGP) used in several MLST and population genetic analyses of *C. hominis* and *C. parvum* (*16–18*) were included in this study. We searched for additional microsatellite and minisatellite markers in the chromosome 6 genome by using Tandem Repeats Finder software (http://tandem.bu.edu/trf/trf.html). Of 325 short tandem repeat sequences identified, 46 loci were initially selected at spaced intervals that covered the entire chromosome 6. A nested PCR approach was used for amplification of all potential targets. After an initial evaluation of primers by PCR and DNA sequencing of IaA15R3, IaA20R3, IbA10G2, IdA10, and IeA11G3T3 subtypes of *C. hominis* from the United States and Peru, 24 polymorphic loci (Table 1, Appendix, wwwnc.cdc.gov/EID/article/19/10/12-1361-T1.htm), and the 8 markers previously identified, were used in this study.

MLST PCR and Sequencing

Conditions for nested PCR analysis of each marker were similar to those described (*16–18*), except for differences in annealing temperatures specified in Table 1. One negative control and 1 positive control (*C. parvum* IIaA16G2R1 subtype) were used in each PCR. PCR products of the expected size were sequenced in both directions by using an ABI 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences obtained from each locus were edited and aligned by using BioEdit 7.04 version (www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalX version 1.81 (www.clustal.org/), respectively. Nucleotide sequences of all haplotypes of polymorphic loci were deposited in GenBank under accession nos. JX088398–JX088417 and JX088427–JX088501.

Analyses of Sequence Polymorphism

For each marker, sequences of 53 *C. hominis* specimens were used for calculation of haplotype diversity (Hd), number of haplotypes, number of polymorphic and segregating sites, intragenic linkage disequilibrium (LD), and intragenic recombination rates by using DnaSP version 5.10.00 (www.ub.es/dnasp/). The ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site was calculated for gp60 and several nearby loci (C6–830′, C6 1000′, C6 1420, CP47, and C6 2600) by using DnaSP. Thereafter, sequences of polymorphic loci were concatenated as a single multilocus contig to calculate gene diversity, interlocus LD, and recombination rates by using DnaSP. Pairwise intergenic LD was also evaluated among polymorphic loci by using the Fisher exact test and Markov chain parameters in Arlequin version 3.1 (http://cmpg.unibe.ch/software/arlequin3/).

Substructure Analysis

Population substructures were analyzed by using the Bayesian analysis tool STRUCTURE version 2.2 (http://pritch.bsd.uchicago.edu/structure.html). Conversion of microsatellite and minisatellite sequences to allelic data was performed by using the Excel Microsatellite Tool Kit 3.1.1 (http://animalgenomics.ucd.ie/sdepark/ms-toolkit/) and Genepop version 4.0 (http://genepop.curtin.edu.au/). Several analyses of allelic data were performed by using K (likely populations) ranging from 2 to 8 and 50,000 iterations after a burn-in of 50,000 iterations. Output at K = 2–5 provided the best fit to MLST data and were used in further analyses. To provide an alternative view of substructuring, a median-joining algorithm analysis (*19*) was conducted by using Network version 4.6.1.0 (www.fluxus-ngineering.com/sharenet.htm). Each gp60 subtype was further analyzed for LD and recombination by using DnaSP and Arlequin. Population differentiation (F_{ST}) between IbA10G2 and non-IbA10G2 subtypes was also assessed at each genetic locus by using Arlequin.

Results

Multilocus Gene Diversity

Sequence polymorphism among the 53 *C. hominis* specimens was detected at 25 loci (Table 2), including variations in copy numbers of microsatellite or minisatellite repeat and single nucleotide substitutions (SNPs) and insertion and deletion (indels) in the nonrepeat regions. The remaining 7 loci (C6–190, C6–500′, TSP8, C6 830, C6 870, MSC6 5, and Mucin1) were monomorphic in the study population. The Hd of individual polymorphic locus ranged from 0.0377 to 0.9231, and most loci with higher Hd were situated in the first 25% of chromosome 6 (Table 2).

Table 2. Intragenic analyses of 53 *Cryptosporidium hominis* specimens at 25 polymorphic loci in chromosome 6*

No.	Locus	Position in chromosome 6	Haplotype diversity	No. haplotypes	Polymorphic sites, bp†	Segregating sites, bp‡	Intragenic LD	Intragenic recombination events
1	CP56	5451–6129	0.4819	3	2	1	NA	NA
2	MSC6–7	12415–12945	0.4819	3	40	1	NA	NA
3	C6–60	22017–22807	0.9231	21	98	3	Y = 1.0000 + 0.0000X	0
4	C6–160	44235–44783	0.6727	3	7	0	NA	NA
5	C6–230	58658–59234	0.4906	6	79	1	NA	NA
6	C6–280	70850–71454	0.5530	3	42	3	Y = 1.0000 + 0.0000X	0
7	C6–350	84688–85277	0.4136	2	21	12	Y = 1.0000 + 0.0000X	0
8	C6–580	138267–138866	0.2409	3	2	2	NA	NA
9	C6–740	188916–189541	0.5791	5	7	1	NA	NA
10	C6–830′	210232–210884	0.1742	2	1	1	NA	NA
11	C6–1000′	251594–252087	0.3832	3	35	20	Y = 1.0000 + 0.0000X	0
12	gp60	266540–267350	0.7090	6	431	297	Y = 0.9729 + 0.0130X	26
13	C6–1420	338725–339242	0.2083	3	13	1	NA	NA
14	CP47	372535–372907	0.8084	6	113	7	Y = 1.0000 + 0.0000X	0
15	C6–2600	606026–606798	0.3570	2	1	1	NA	NA
16	C6–2970	685852–686541	0.3570	2	6	2	NA	NA
17	C6–3110′	721467–722169	0.0377	2	9	0	NA	NA
18	C6–3520	820500–821215	0.4136	2	6	0	NA	NA
19	C6–3520′	822524–823076	0.0377	2	1	1	NA	NA
20	C6–3690	864809–865416	0.4688	2	1	1	NA	NA
21	DZ-HRGP	917600–918139	0.1742	2	3	0	NA	NA
22	C6–4110	956461–957083	0.4115	4	27	5	Y = 1.0000 + 0.0000X	0
23	C6–5110′	1206378–1206774	0.2663	3	8	2	NA	NA
24	C6–5120	1213134–1213677	0.2612	2	6	0	NA	NA
25	C6–5410	1285377–1286003	0.5218	3	9	0	NA	NA

*LD, linkage disequilibrium, where Y is the LD value, and X is the nucleotide distance in kilobases; NA, analysis is not applicable for the locus, which has <3 segregating sites.
†Polymorphic sites including insertions/deletions (indels).
‡Segregating sites excluding indels.

Number of haplotypes per locus ranged from 2 to 21, and more haplotypes were at loci C6–60 (21), CP47 (6), gp60 (6), C6–230 (6), and C6–740 (5). MLST analysis confirmed the absence of concurrence of mixed *C. hominis* subtypes in the 53 specimens.

Alignment of combined multilocus sequences covered 15,717 bp and 968 polymorphic sites, including 362 segregating sites and 606 indels. Multilocus sequences had 43 multilocus genotypes (MLGs), an Hd of 0.9898, and a nucleotide diversity of 0.339. The frequency of MLGs ranged from 7.5% (1 MLG with 4 specimens), 5.7% (1 MLG with 3 specimens), and 3.8% (5 MLGs each with 2 specimens) to 1.9% (36 MLGs each with 1 specimen). Because 431/968 polymorphic sites and 297/362 segregating sites occurred within the gp60 locus, a second analysis was performed by using concatenated sequences excluding gp60 (14,768 bp) with 537 polymorphic sites, including 65 segregating sites and 472 indel sites. A lower nucleotide diversity of 0.289 was observed. However, the number of MLGs (43) and haplotype diversity (0.9898) remained the same.

LD and Recombination

Intragenic LD between pairs of segregating sites was assessed for each polymorphic locus. The analysis was possible only for 7 loci (C6–60, C6–280, C6–350, C6–1000′, gp60, CP47, and C6 4110) that had at ≥3 segregating sites. Incomplete intragenic LD (|D′| Y = 0.9729 + 0.0130X), where Y is the LD value and X is the nucleotide distance in kb, was observed at the gp60 locus, and complete intragenic LD (|D′| Y = 1.0000 + 0.0000X) was found at each of the 6 remaining loci (Table 2). An intragenic recombination test identified 26 potential recombination events (Rms) at the gp60 locus and no recombination at the remaining loci (Table 2).

On the basis of the concatenated multilocus sequence data, interlocus LD was assessed over all segregating sites by using pairwise comparisons (*20*). Analysis of combined multilocus sequences of all loci resulted in an overall interlocus genetic association ($Z_n s$) value of 0.2711 (95% CI 0.0695–0.3918; the probability p for expected $Z_n s \leq 0.2711$ was 0.883). Of 47,895 pairwise comparisons, 25,904 were significant by Fisher exact test, and 8,714 were significant after Bonferroni correction (Table 3).

Table 3. Pairwise interlocus linkage disequilibrium and recombination analysis of concatenated multilocus sequences from various subtypes of *Cryptosporidium hominis**

Population	No. samples	No. segregating sites analyzed	No. pairwise comparisons	No. significant pairwise comparisons†	$Z_n s$	|D'|	LD	Estimate of R/gene	Minimum no. recombination events
Including gp60‡									
All	53	362	47,895	25,904 (8,714)	0.2711	Y = 0.9289 – 0.0299X	Inc	5.0	33
IaA13R8(7)	10	20	190	0	0.7581	Y = 1.0000 + 0.0000X	Com	0.001	0
IbA10G2	26	31	465	160 (140)	0.3660	Y = 0.9342 + 0.0001X	Inc	0.3	4
IdA10	6	14	91	0	1.0000	Y = 1.0000 + 0.0000X	Com	0.001	0
IdA20	5	3	3	0	0.3750	Y = 1.0000 + 0.0000X	Com	0.001	0
IeA11G3T3	6	19	171	0	1.0000	Y = 1.0000 + 0.0000X	Com	0.001	0
Excluding gp60§									
All	53	65	2080	921 (333)	0.2184	Y = 0.7886 + 0.0051X	Inc	1.8	6
IaA13R8(7)	10	20	190	0	0.7581	Y = 1.0000 + 0.0000X	Com	0.001	0
IbA10G2	26	31	465	160 (140)	0.3660	Y = 0.9302 + 0.0010X	Inc	0.3	4
IdA10	6	14	91	0	1.0000	Y = 1.0000 + 0.0000X	Com	0.001	0
IdA20	5	3	3	0	0.3750	Y = 1.0000 + 0.0000X	Com	0.001	0
IeA11G3T3	6	19	171	0	1.0000	Y = 1.0000 + 0.0000X	Com	0.001	0

*$Z_n s$, interlocus genetic association; |D'|, linkage disequilibrium (LD) value, where Y is LD value and X is nucleotide distance in kilobases; gp 60, 60-kDa glycoprotein gene; Inc, incomplete; Com, complete.
†By Fisher exact test (after Bonferroni correction).
‡Based on concatenated multilocus gene sequence of all loci (15,717 bp).
§Based on concatenated multilocus gene sequence of all loci excluding gp60 (14,768 bp).

In an additional LD analysis per site, strong but incomplete LD (|D′| Y = 0.9289–0.0299X) was detected with a negative slope, indicating a decrease in linkage with increased nucleotide distance (Table 3). When analysis was performed after excluding gp60, the test produced a $Z_n s$ value of 0.2184 (95% CI 0.0654–0.4469, p = 0.759). Of 2,080 pairwise comparisons, 921 were significant by Fisher exact test, and 333 were significant after Bonferroni correction. Incomplete LD (|D′| Y = 0.7886 + 0.0051X) was also observed in LD analysis per site (Table 3). Thus, recombination might occur because of incomplete LD. An overall recombination test showed a minimum of 33 potential recombination events and an estimated 5.0 R/gene (Table 3). When analysis was performed after exclusion of gp60, only 6 recombination events and 1.8 R/gene were observed (Table 3). Thus, recombination was occurring mostly at the gp60 locus.

Substructure in *C. hominis*

The evolutional relationship among gp60 subtypes of *C. hominis* was inferred by using STUCTURE and predicted population numbers K = 2–5. The ancestral population size K = 5 was considered the best estimate of current population substructure (Figure 1). The most dominant feature in the output was heterogeneity of specimens belonging to subtype IbA10G2 compared with other subtypes. Within 26 specimens of IbA10G2, the pattern of combinations in STRUCTURE suggested a mixture of ancestral types, reflecting likely genetic recombination in the subtype. In contrast, the 10 specimens of the Ia subtype (including 9 specimens of IaA13R8 and 1 specimen of IaA13R7) had mostly a single pattern, thus providing strong support for it being a separate population with a unique ancestry. Likewise, intrasubtype homogeneity was also observed in 6 specimens of IdA10, five specimens of IdA20, and 6 specimens of IeA11G3T3. Within the Id subtype, specimens of IdA10 and IdA20 showed different patterns regardless of the K value, indicating that these 2 Id subtypes had distinct ancestries with little mixture and genetic recombination. On the basis of patterns at K = 5 (Figure 1), some IbA10G2 specimens had a mixture of patterns, which were the dominant patterns in subtype IaA13R8 and subtype IeA11G3T3, respectively, suggesting that these IbA10G2 specimens might have resulted from genetic exchange between the ancestor of subtype IbA10G2 and the ancestor of subtype IaA13R8 or IeA11G3T3.

We also conducted a median-joining network analysis of the MLST data for the 53 specimens. Among 21 MLGs

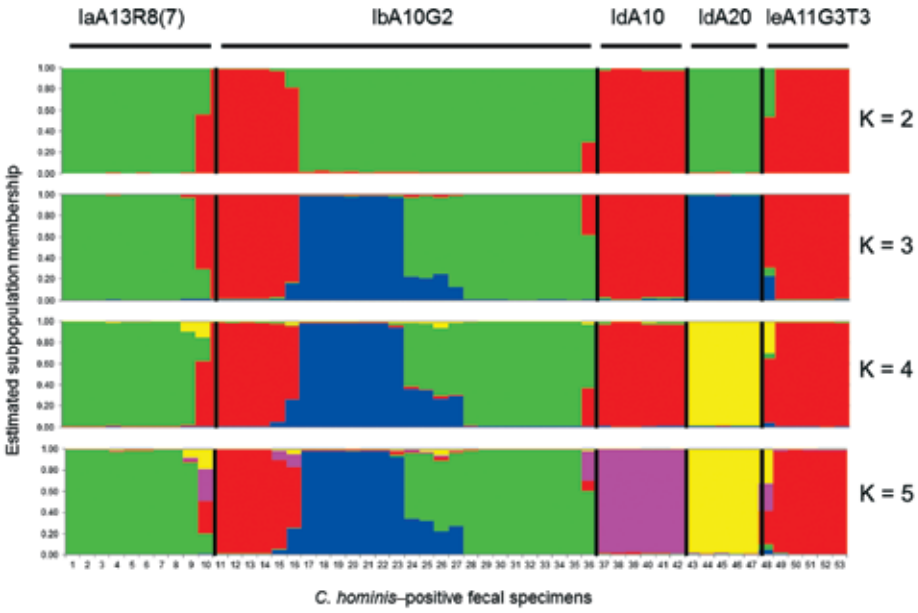

Figure 1. Relationship among various 60-kDa glycoprotein gene subtypes of *Cryptosporidium hominis* by substructure analysis. Predicted population numbers K = 2–5 were applied in STRUCTURE version 2.2 (http://pritch.bsd.uchicago.edu/structure.html) analysis of the data. Colored regions indicate major ancestral contributions. Mixed genotypes are indicated by the pattern of color combinations. Values along the baseline indicate *C. hominis*–positive fecal specimens.

generated on the basis of segregating sites of concatenated sequences, excluding gp60, we did not find any shared MLGs between gp60 subtypes of *C. hominis* (Figure 2). Compared with STRUCTURE analyses, the results of network analysis showed similarity to patterns at K = 2 (Figure 1), and supported the conclusion on the heterogeneity of subtype IbA10G2. In network analysis, central types are usually possible ancestors, and peripheral types are descendants (*21*). Because we did not find any MLG as the central type, it was impossible to define a single ancestral line that gave rise to other lines. This finding could be caused by the small sample size and few MLGs in this study.

Comparative Population Genetics of IbA10G2

The population genetics of various gp60 subtypes was assessed by analyses of LD and recombination rates. In pairwise interlocus LD analysis of multilocus sequences including or excluding gp60, strong but incomplete LD ($|D'|$ $Y = 0.9342 + 0.0001X$ or $|D'|$ $Y = 0.9302 + 0.0010X$) was observed in subtype IbA10G2, suggesting recombination within this subtype. In contrast, all other subtypes showed complete LD ($|D'|$ $Y = 1.0000 + 0.0000X$) among all sites, indicative of no genetic recombination within these subtypes (Table 3). In addition, pairwise intergenic LD was also evaluated between 25 loci, resulting in 87 instances

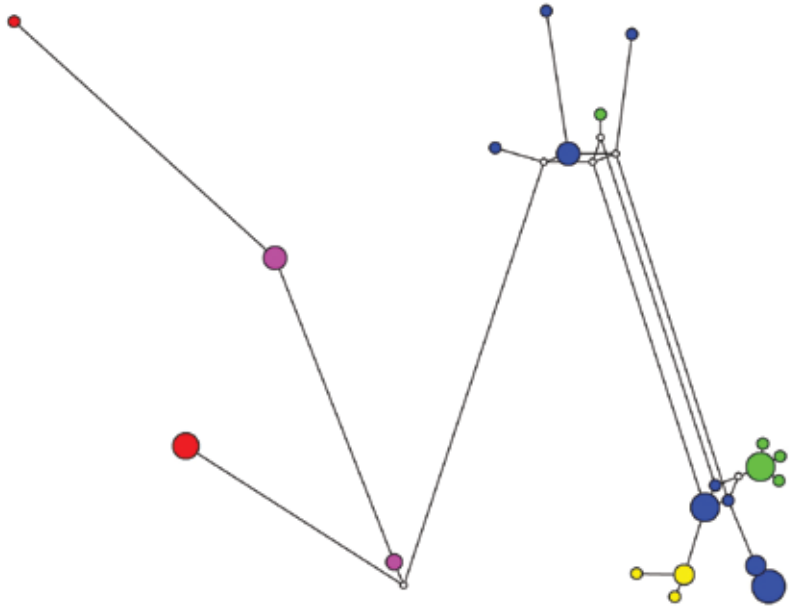

Figure 2. Median-joining network for various subtypes of *Cryptosporidium hominis*. Circles are proportional to the frequency of each multilocus genotype (MLG) (21 MLGs on the basis of segregating sites of concatenated sequences excluding the 60-kDa glycoprotein gene). The color of each circle represents the 60-kDa glycoprotein gene subtypes of the *C. hominis* specimens: IaA13R8 (green), IbA10G2 (blue), IdA10 (purple), IdA20 (yellow), and IeA11G3T3 (red). Length of lines connecting MLGs is proportional to the number of single-nucleotide polymorphisms.

of significant pairwise LD ($p<0.05$) observed in subtype IbA10G2 compared with 227 significant LD in the remaining subtypes (Figure 3). In IbA10G2 specimens, loci around gp60 (locus 12) had no LD with any other loci in chromosome 6. This finding indicated genetic recombination in subtype IbA10G2 at loci near gp60.

To validate these observations, recombination tests were conducted for all gp60 subtypes. Using full multilocus sequences, we found that among 33 potential Rms observed in overall recombination analysis, 4 Rms were detected in subtype IbA10G2 but no Rm was found within each of the remaining subtypes (Table 3). When analysis was performed that excluded gp60, among 6 potential Rms in the overall recombination analysis, 4 Rms were still detected in subtype IbA10G2 but no Rm was seen in the other subtypes (Table 3). Thus, genetic recombination occurred only within the subtype IbA10G2, a conclusion in agreement with results of the STRUCTURE and LD analyses.

The genetic determinant for differences between IbA10G2 and non-IbA10G2 subtypes was assessed by comparison of haplotype diversity of the 2 groups at all 25 polymorphic loci in chromosome 6. At most of the genetic loci, the haplotype diversity of the 2 groups was similar, and there was no clear population differentiation at these loci (Figure 4, Table 4). However, at 4 loci (C6–830′, C6 1000′, gp60, C6 1420) around gp60, an absence of genetic diversity was observed in subtype IbA10G2 compared with non-IbA10G2 subtypes (Figure 4). The region (129 kb) of homogeneity in subtype IbA10G2 was located in a 210–339-kb region in chromosome 6. Pairwise F_{ST} analysis showed highly significant differentiation between the IbA10G2 subtype and non-IbA10G2 subtypes at 4 loci around gp60, C6–1000′ ($p<0.00001$), gp60 ($p<0.00001$), CP47 ($p<0.00001$), and C6 2600 ($p<0.001$). The only other locus that showed such a level of differentiation between the 2 groups was C6–3520 (Table 4).

Discussion

Like other apicomplexan parasites, the life cycle of *Cryptosporidium* spp. has a sexual phase, during which sexual recombination can occur between genetically distinct strains (*22*). *C. parvum*, the species that infects humans and some animals, undergoes meiotic recombination between different lineages in genetic-crossing experiments (*23*,*24*). LD analyses of natural *C. parvum* populations have also shown genetic recombination in most study areas (*22*,*25–28*). In contrast, *C. hominis* is primarily infectious to humans, and previous MLST studies showed a clonal population structure. Genetic recombination was believed to be rare or nonexistent in *C. hominis* (*17*,*25*,*28–30*). However, the small number of markers used in previous studies might have resulted in relatively low resolution in population structure analysis, which could have led to failure in detecting genetic recombination in *C. hominis*. In the present study, we examined population substructure of *C. hominis* in a cohort of children living in a small study area by using 32 genetic markers.

On the basis of multilocus sequence data and allelic profiles for 53 specimens, analysis showed strong LD among 25 polymorphic loci, suggesting an overall nonpanmictic population structure of *C. hominis*. A recombination test showed only limited genetic recombination at the gp60 locus. Thus, the high level of LD and limited recombination found in the overall population could be explained

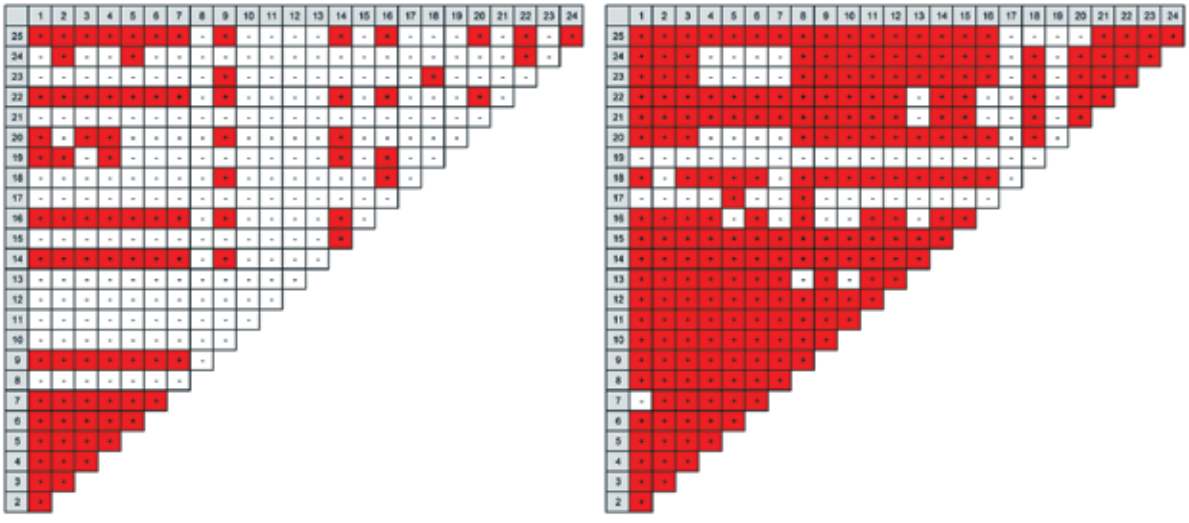

Figure 3. Pairwise intergenic linkage disequilibrium (LD) matrix among 25 polymorphic loci of *Cryptosporidium hominis*. The 25 polymorphic loci are indicated in gray (see Table 2 for identification of loci). Significant LD between loci is indicated in red. Subtype IbA10G2 (A), which has 87 instances of pairwise LD, has fewer LD in comparison with the remaining subtypes (B), which have 227 instances of pairwise LD. In IbA10G2, loci around the 60-kDa glycoprotein gene (locus 12) have no LD with any other loci in chromosome 6.

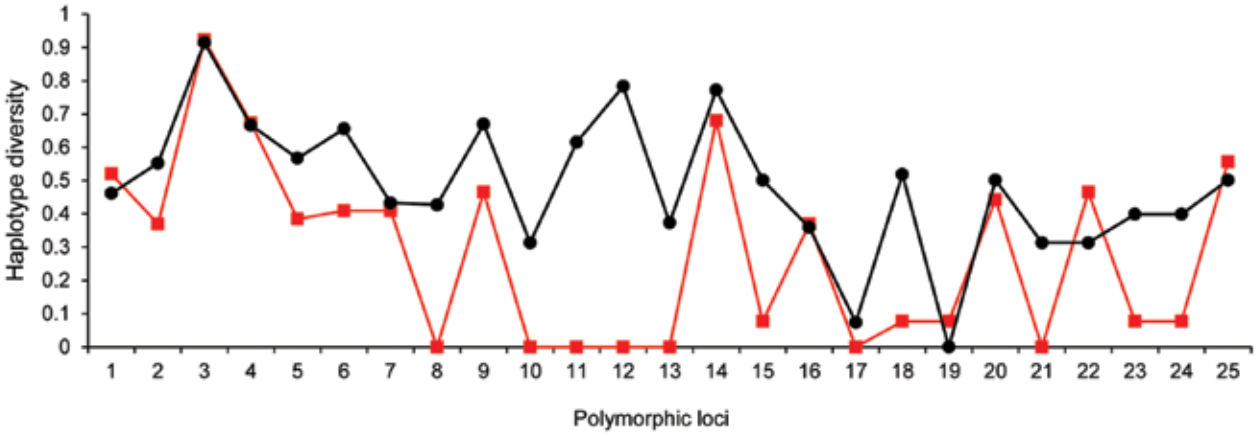

Figure 4. Genetic variation in haplotype diversity at 25 polymorphic loci in chromosome 6 of *Cryptosporidium hominis* (see Table 2 for identification of loci). Red squares indicate subtype IbA10G2 and black circles indicate non-IbA10G2 subtypes. A homogeneity (reduced haplotype diversity) of subtype IbA10G2 was seen in 4 loci around the 60-kDa glycoprotein gene.

by an essential clonal population structure of *C. hominis* in the field site in Peru, which is consistent with information on *C. hominis* population genetics in other countries (*17*,*25*,*28*,*29*).

Results of LD and recombination analyses suggest that limited recombination in the study population of *C. hominis* occurred mostly at gp60 or loci around gp60. Thus far, the gp60 gene is the most polymorphic marker identified in the *Cryptosporidium* genome. Because of its high sequence heterogeneity, gp60 has become the most widely used gene in *Cryptosporidium* spp. subtyping, which categorizes *C. hominis* and *C. parvum* into several subtype groups and various subtypes within each subtype group (*2*). The gp60 gene encodes glycoproteins gp15 and gp45, which are implicated in attachment to and invasion of host cells (*31*,*32*). Because attachment of sporozoites to epithelial cells and invasion of the host cell membrane are crucial steps in the pathogenesis of cryptosporidiosis, these 2 glycoproteins are presumed to be surface-associated virulence determinants that may be under host immune selection, which might explain the extensive polymorphism in the gp60 gene (*27*). Genetic recombination appears to be associated with high sequence polymorphism in the gp60 gene (*27*). However, a less speculative understanding of the role the gp60 gene in pathogenesis of cryptosporidiosis requires further investigations.

Recent studies have suggested that telomeric/subtelomeric regions are highly polymorphic and might encode putative virulence factors (*33*,*34*). However, these studies did not compare phenotypic differences among isolates, and data for the present study do not relate directly to sequence variations at telomeres. In the present study, because *C. hominis* subtype IbA10G2 was shown to be more virulent than other subtypes in the study community (*12*), we compared population genetics of IbA10G2 and other gp60 subtypes. Heterogeneity within subtype IbA10G2 was observed in STRUCTURE and Network analyses compared with homogeneity within each of the remaining subtypes. This finding suggested a mixture of ancestral genetic elements and genetic recombination in virulent subtype IbA10G2. This finding was confirmed by incomplete LD and several recombination events (4 Rms) found in IbA10G2. In addition, the pattern of shared ancestral types in this study suggests that genetic exchange might have occurred between the ancestor of subtype IbA10G2 and the ancestor of subtype IaA13R8 or IeA11G3T3.

Pairwise F_{ST} analysis of 25 polymorphic loci in chromosome 6 between the virulent IbA10G2 subtype and non-IbA10G2 subtypes showed population differentiation at 4 loci around gp60. When a locus shows extraordinary levels of genetic population differentiation compared with other loci, this finding might be interpreted as evidence for positive selection (*35*). Thus, the region around gp60 in subtype IbA10G2 might be under selection pressure. This finding was further confirmed by comparison of haplotype diversity of the 2 groups at the 25 polymorphic loci. Although a similar Hd was found between the 2 groups in most regions of chromosome 6, a region of Hd reduction was observed in subtype IbA10G2 in a 129-kb region flanking gp60, compared with the non-IbA10G2 subtypes.

One explanation for this pattern of genetic diversity is that the region surrounding gp60 was probably affected by selective sweep or genetic hitchhiking caused by selection of the virulence subtype. A hallmark of a selective sweep is a chromosomal region with reduced diversity associated with a specific phenotype. In previous studies of other apicomplexan parasites, similar patterns of reduced genetic variations were observed in chromosomal regions

Table 4. Estimates of haplotype diversity in IbA10G2subtype and non-IbA10G2 subtypes of *Cryptosporidium hominis* and comparison of 2 populations at each locus by pairwise F_{ST} analysis*

No.	Locus	Haplotype diversity		Population pairwise	
		IbA10G2 subtype	Non-IbA10G2 subtypes	F_{ST}	p value ± SEM
1	CP56	0.5200	0.4615	–0.03454	0.88867 ± 0.0104
2	MSC6–7	0.3692	0.5527	0.07491	0.10156 ± 0.0074
3	C6–60	0.9231	0.9145	0.00922	0.21387 ± 0.0105
4	C6–160	0.6738	0.6667	0.00735	0.31738 ± 0.0131
5	C6–230	0.3846	0.5670	0.05070	0.07422 ± 0.0069
6	C6–280	0.4092	0.6553	0.06300	0.06836 ± 0.0076
7	C6–350	0.4092	0.4330	–0.03736	0.99902 ± 0.0002
8	C6–580	0.0000	0.4274	0.17205	0.00781 ± 0.0028†
9	C6–740	0.4646	0.6695	0.03343	0.12891 ± 0.0126
10	C6–830′	0.0000	0.3134	0.15031	0.04883 ± 0.0067‡
11	C6–1000′	0.0000	0.6154	0.30288	0.00000 ± 0.0000§
12	gp60	0.0000	0.7835	0.60340	0.00000 ± 0.0000§
13	C6–1420	0.0000	0.3732	0.15665	0.02051 ± 0.0038‡
14	CP47	0.6800	0.7721	0.18033	0.00000 ± 0.0000§
15	C6–2600	0.0769	0.5014	0.29888	0.00098 ± 0.0010§
16	C6–2970	0.3692	0.3590	–0.03901	0.99902 ± 0.0002
17	C6–3110′	0.0000	0.0741	–0.00143	0.99902 ± 0.0002
18	C6–3520	0.0769	0.5185	0.42036	0.00000 ± 0.0000§
19	C6–3520′	0.0769	0.0000	0.00148	0.48535 ± 0.0148
20	C6–3690	0.4431	0.5014	–0.01700	0.55859 ± 0.0145
21	DZ-HRGP	0.0000	0.3134	0.15031	0.03809 ± 0.0056‡
22	C6–4110	0.4646	0.3134	0.10817	0.01855 ± 0.0048‡
23	C6–5110′	0.0769	0.3989	0.17072	0.00781 ± 0.0028†
24	C6–5120	0.0769	0.3989	0.14126	0.05664 ± 0.0066
25	C6–5410	0.5569	0.5014	–0.02614	0.67383 ± 0.0182

*F_{ST}, population differentiation.
†p<0.01.
‡p<0.05.
§p<0.001.

surrounding sites under selection pressure. Wootton et al. (*36*) found a dramatic reduction in genetic variation in chloroquine-resistant parasites within a region spanning >200 kb around the *Plasmodium falciparum* chloroquine-resistance transporter gene in chromosome 7 as the result of selection for chloroquine resistance. Nair et al. (*37*) observed decreased variation in an ≈100-kb region flanking the dihydrofolate reductase gene in chromosome 4 of *P. falciparum* in association with pyrimethamine resistance. Consistent with these findings, we detected reduced sequence variation around gp60 in virulent subtype IbA10G2, suggesting that the 129-kb region surrounding gp60 in chromosome 6, perhaps gp60 itself, might be involved in selection for virulent gp60 subtype IbA10G2 in *C. hominis*. In a comparison of the number of nonsynonymous substitutions per nonsynonymous site to the number of synonymous substitutions per synonymous site analysis of loci around gp60, we confirmed the presence of positive section at least at the CP47 locus.

The reason for exclusive occurrence of genetic recombination in IbA10G2 is not clear. Because IbA10G2 is the major subtype of *C. hominis* responsible for numerous waterborne and foodborne outbreaks of cryptosporidiosis in many countries (*2*), it is transmitted frequently among humans, resulting in increased probability of mixed infections with other genetically heterogeneous subtypes, especially in countries in Europe in which autochthonous *C. hominis* infections are caused mostly by IbA10G2 and imported cases by other subtypes (*38*). In a long evolutionary process, the common occurrence and biologic fitness of the gp60 IbA10G2 subtype may facilitate genetic recombination with other *C. hominis* subtypes and subsequent spread of the recombinant parasite with the fitness gene. Recently, genetic recombination was shown to be a key strategy for selection of virulent clones of *Toxoplasma gondii*, an apicomplexan parasite with a largely clonal population structure in North America and Europe (*39*).

In conclusion, we have shown complex substructures in a natural *C. hominis* population in a cohort of children living in a small community in Peru. Although *C. hominis* from the community has an overall clonal population structure, genetic recombination occurs within subtype IbA10G2 around the gp60 locus, which might be involved in pathogenicity. Common occurrence of its parental subtypes and biologic fitness of the recombinant subtype with the IbA10G2 trait have probably facilitated genetic

exchange and spread of the virulent subtype. In addition, we were able to localize selection for the virulent subtype IbA10G2 to a 129-kb region surrounding gp60 in chromosome 6. These observations could improve our understanding of emergence and spread of virulent *C. hominis* subtypes.

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Emergence of Vaccine-derived Polioviruses, Democratic Republic of Congo, 2004–2011

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Polioviruses isolated from 70 acute flaccid paralysis patients from the Democratic Republic of Congo (DRC) during 2004–2011 were characterized and found to be vaccine-derived type 2 polioviruses (VDPV2s). Partial genomic sequencing of the isolates revealed nucleotide sequence divergence of up to 3.5% in the viral protein 1 capsid region of the viral genome relative to the Sabin vaccine strain. Genetic analysis identified at least 7 circulating lineages localized to specific geographic regions. Multiple independent events of VDPV2 emergence occurred throughout DRC during this 7-year period. During 2010–2011, VDPV2 circulation in eastern DRC occurred in an area distinct from that of wild poliovirus circulation, whereas VDPV2 circulation in the southwestern part of DRC (in Kasai Occidental) occurred within the larger region of wild poliovirus circulation.

Indigenous wild-type poliovirus (WPV) remains endemic to 3 countries: Nigeria, Afghanistan, and Pakistan (*1*). Poliovirus (PV) circulation has been sustained in several African countries after importation from a PV-endemic country, resulting in reestablished virus transmission. Worldwide, the number of cases decreased by 50% from 2010 to 2011 (*2*). In developing countries, live attenuated oral PV vaccine (OPV) is still the vaccine of choice. However, the virus can revert to virulence during OPV replication in humans, resulting in person-to-person spread and circulation of vaccine-derived PVs in areas with low rates of vaccination coverage (*3*). Substantial sequence drift occurs in circulating VDPVs (>1% nt difference in types 1 and 3, >0.6% nt difference in type 2), indicating prolonged replication of the vaccine strain in human populations and consequent changes in phenotypic properties of neurovirulence and transmissibility (*3*,*4*). Poliomyelitis outbreaks associated with circulating VDPVs have been reported in several countries, including Egypt (1982–1993), Haiti (2000–2001), Dominican Republic (2000–2001), Philippines (2001), Madagascar (2002 and 2005), China (2004), Cambodia (2005–2006), Indonesia (2005), and Nigeria 2005–2010 (*4–11*). As a result of accumulating evidence about the emergence and spread of circulating VDPVs, there are plans to stop using OPV and synchronously implement more widespread use of inactivated polio vaccine (*12–15*). A better understanding of VDPV persistence and circulation is crucial for deciding when and how to stop vaccination with OPV after WPVs have been eradicated (*16–18*).

In early 2001, indigenous WPVs were eliminated from DRC, but starting in 2006 and continuing through 2011, WPV was imported into DRC from Angola several times. During 2010-2011 in DRC, 2 genetic clusters of the Southeast Asian PV1 genotype circulated; this genotype was imported twice from India to Angola and subsequently to DRC. Since December 2011, no cases of infection with WPV have been detected in DRC (*19*).

Monovalent OPV types 1 and 3 and bivalent OPV effectively induce immunity because of a lack of interference by the type 2 component (*20*). Reliance on monovalent OPV1, monovalent OPV3, and bivalent OPV in supplemental vaccination activities has contributed to the emergence of VDPV2. These alternative OPV formulations are

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more effective than trivalent OPV at inducing higher levels of population immunity to WPV1 and WPV3 because there is no interference from the type 2 OPV strain. No type 2–specific immunity is induced. To maintain population immunity to type 2 PV, the World Health Organization (WHO) recommends 2 doses of trivalent OPV each year.

We describe the genetic characterization of circulating VDPV2 associated with outbreaks in DRC. During 2004–2011, the same time that extensive circulating VDPV transmission occurred in Nigeria, VDPV2 excretion was found for 70 children with acute flaccid paralysis (*10*,*11*). The close genetic relationships among many of the viruses provide evidence for circulation in several regions of DRC.

Materials and Methods

Viruses

National authorities in DRC submitted fecal specimens from patients with acute flaccid paralysis to the National Institute for Biomedical Research, Kinshasa, for PV isolation by standard methods recommended by WHO. To determine serotype of PV isolates and whether the virus was wild or related to vaccine strains (known as intratypic differentiation), isolates were forwarded to the National Institute for Communicable Diseases in South Africa for characterization by PCR, ELISA, and partial genomic sequencing (*21*). The original fecal specimens from which these isolates were obtained were also sent to the National Institute for Communicable Diseases for confirmation of virus isolation results by methods recommended by WHO (*22*).

Intratypic Differentiation

Through use of PCR (*21*–*23*) and ELISA, as recommended by WHO, PV isolates were determined to be Sabin-like or non–Sabin-like strains (*23*–*26*). All serotyped PVs or virus isolates that had shown cytopathic effect in L20B cells were tested by using a reverse transcription PCR (RT-PCR) kit supplied by the Centers for Disease Control and Prevention (Atlanta, GA, USA), which included separate reactions with primers for panenterovirus; panpoliovirus; PV serotypes 1, 2, and 3 (*23*,*24*); and multiplexed primers for Sabin type 1, 2, and 3 PVs (*25*). The amplicons were separated on 10% polyacrylamide gels and visualized after staining with ethidium bromide. Additionally, serotyped PV monotypes were analyzed by using an ELISA developed by the National Institute for Public Health and the Environment (Bilthoven, the Netherlands) and identified as Sabin-like or non–Sabin-like strains by using specific cross-adsorbed antiserum (*21*). From 2007 on, a real-time PCR was used for intratypic differentiation of untyped L20B isolates with a positive cytopathic effect. Another real-time PCR was used for retrospective VDPV screening of Sabin strains reported during 2004-2006 and for prospective screening of untyped strains isolated from 2009 on (*27*,*28*).

Sequencing

RNA was extracted from 140 μL of cell culture supernatant by using a QIAamp viral RNA extraction kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's recommendations. RT-PCR was performed in a single step, as described (*29*). Briefly, the extracted RNA (10 μL) was added to 90 μL of the amplification mixture containing 10 μL standard 10× reaction buffer, 100 μM of each dNTP (Roche Diagnostics GmbH, Mannheim, Germany), 10 mM dithiothreitol, 80 pmol of each primer (Q8 and Y7), 20 U of placental RNase inhibitor (Roche Diagnostics GmbH), 12.5 U of avian myeloblastosis virus reverse transcriptase (Roche Diagnostics GmbH), and 5U of Taq DNA polymerase (Roche Diagnostics GmbH). RT was conducted at 42°C for 60 min in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA, USA) followed by denaturation at 95°C for 3 min. Amplification consisted of 30 cycles (95°C for 30 sec, 42°C for 30 sec, and 60°C for 2 min). The amplicons were visualized by ethidium bromide staining after electrophoresis on 1.5% agarose gels as described (*26*).

Before sequencing, the RT-PCR products were purified with the QIAquick PCR purification kit (QIAGEN). The complete viral protein (VP) 1 gene (nt 2480–3385) was sequenced as described (*29*,*30*) by cycle sequencing with the BigDye Terminator version 3.1 Cycle Sequencing Kit (*31*) (Applied Biosystems). The DNA sequence was determined by using the ABI 3100 Genetic Analyzer, version 3.1 (Applied Biosystems). Raw data files were imported into the Sequencher software package version 4.1.4 (http://avaxho.mc/software/soaftware_type/scientific/others/sequencher_414.htm) for assembly and editing, and consensus sequence files were produced.

Sequence Analysis

According to WHO guidelines, Sabin viruses that differ from Sabin 2 by >5 nt in the VP1 coding region are classified as VDPVs (*4*). To determine VP1 genetic diversity, we compared all complete VP1 sequences of VDPV2 isolates from patients with acute flaccid paralysis and their contacts with the sequence of the Sabin type 2 OPV reference strain (GenBank accession no. AY184220). Evolutionary distances were computed by using the Kimura 2-parameter method (*32*) and the neighbor-joining method (*33*,*34*). Phylogenetic trees were constructed by using MEGA5 (*35*) with 500 bootstrap replicates. Bootstrap values >80 (out of 100) are indicated on the tree.

The alignments (nucleotide and translated amino acid sequences) were analyzed by using GeneDoc version

2.6001 (www.psc.edu/biomed/genedoc) and MEGAs 5 (*35*) to identify specific mutations and positive selection. Assignment of isolates to independent VDPV2 emergences and lineage groups was based on pairwise VP1 capsid region sequence differences within and between lineage groups. Complete capsid region sequence differences were used when there was uncertainty about relationships that had been based on the VP1 region; complete genome sequences were used to identify possible recombination junctions (data not shown).

Results

Circulating VDPVs

In total, just over 600 viral isolates obtained from DRC during 2004–2011 were tested by RT-PCR by using panenterovirus; panpoliovirus; serotype-specific; and Sabin type 1, 2, and 3 virus–specific primers. Isolates received before implementation of diagnostic real-time PCR were further tested by ELISA, and both techniques identified the isolates as Sabin-like PVs. All isolates were further screened by using real-time RT-PCR, as has been implemented in the Global Polio Laboratory Network, which included a screen for VDPVs (*9*). The genetic variability of virus isolates was further investigated by performing nucleotide sequence analysis of the VP1 capsid region of isolates that were identified as possible VDPVs by the VDPV screening assay. Partial genomic sequencing confirmed that for 70 cases, VP1 sequence was ≤99.3% identical to that of the parental Sabin serotype 2 strain (i.e., ≥6 nt substitutions), and the isolates were classified as VDPVs. In all isolates, VP1 amino acid changes associated with the reversion to virulence (*3*) were identified at position 143.

During 2004–2011, VDPV2s were isolated from fecal specimens from patients with acute flaccid paralysis in several regions of DRC (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0028-Techapp1.pdf). The first case occurred in Maniema Province, in Kindu District, on October 13, 2004. For the other provinces, 40 cases occurred in Katanga, 12 in Maniema, 6 in Orientale, 5 in Kasai Occidental, 4 in Équateur, 2 in Bandundu, and 1 in Sud Kivu (Figures 1 and 2). During 2010–2011, a total of 193 cases of WPV1 were also reported in DRC; some of the geographic areas where VDPVs were found overlapped. During this time, ongoing reestablished transmission of WPV1 in the eastern part of the country occurred in northeastern Katanga, whereas VDPVs were found in central Katanga. In 2010, in Kasai Occidental, southwestern DRC, a different genetic cluster of WPV1 was introduced from Angola and the Republic of Congo. The extensive area of WPV1 circulation overlapped with the more restricted area of circulation of VDPVs in Kasai Occidental (*19*).

Phylogeny of the VP1 Region of the VDPVs

Emergence of VDPV2 was first detected in Maniema Province in October 2004. No other related viruses were

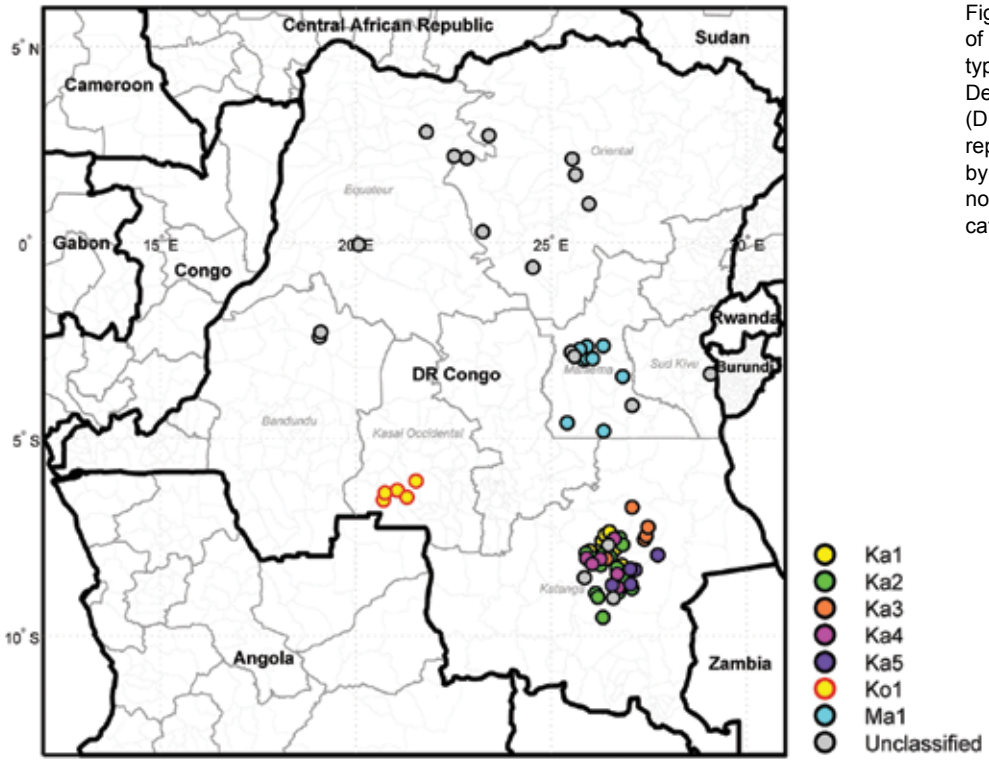

Figure 1. Geographic distribution of vaccine-derived polivirus type 2 from patients from the Democratic Republic of Congo (DR Congo). Viruses are represented by circles colored by lineage. Viruses that are not assigned to a lineage are categorized as unclassified.

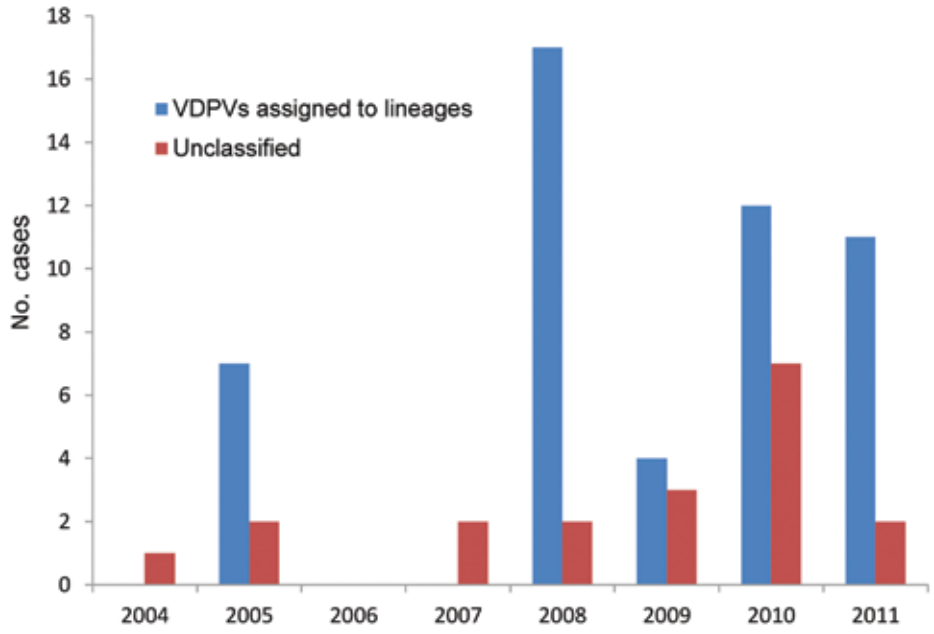

Figure 2. Yearly incidence of vaccine-derived polivirus type 2, Democratic Republic of Congo, 2004–2011. The total number of cases associated with vaccine-derived polivirus type 2 is graphed for each year according to date of onset of paralysis. Viruses that are not assigned to a lineage are categorized as unclassified. VDVPs, vaccine-derived PVs.

detected (Figure 3). Emergence of VDPV was next detected in Katanga in July 2005 (Figure 3). Four additional polio cases with onset during the same month were identified in Katanga, and 2 others followed in August. Viruses from the patients with polio onset in July and August were closely related to each other, forming lineage Ka1, which circulated in the district of Kinkondja in Katanga. Circulation of this lineage apparently stopped abruptly in 2005; no additional related viruses were detected in later years. The other 4 viruses isolated during 2005 and 2007 represented independent VDPV emergence events in 3 provinces (Bandundu, Sud-Kivu, and Orientale).

In 2008, two concurrent VDPV2 outbreaks (lineages Ka2 and Ka3) were detected in Katanga (Figure 3). Both outbreaks continued into 2009 in central Katanga. Also in 2008, two individual VDPV emergence events were detected in Maniema and Orientale.

In 2009, an outbreak in Kasai Occidental was detected and continued into 2010 (Ko1 lineage). In June 2010, an outbreak was detected in Maniema Province in several districts (Ma1 lineage); the last case occurred in October 2010. In addition, in 2009 and 2010, individual VDPVs representing independent emergence events were detected in Equateur, Maniema, Oriental, and Katanga Provinces.

In late 2011, several VDPVs were detected in Katanga; 4 independent events of VDPV emergence were observed, 2 of which formed lineages consisting of 5 or 6 VDPVs with 0.6%–1.2% nt difference from Sabin 2 (Ka4 and Ka5). During the 7-year period, the Katanga outbreaks accounted for 40 VDPVs, which is more than half of the total number of VDPVs detected in DRC.

Discussion

We isolated VDPVs from fecal specimens from 70 patients with acute flaccid paralysis in DRC during 2004–2011. Sequence analysis of the VP1 coding region of these viruses showed that 51 isolates represented 7 circulating lineages of circulating VDPVs, and 19 others represented independent emergence events. The detection of several distinct circulating VDPV lineages in DRC reinforces previous observations that circulating VDPVs can emerge independently in locations where immunity to PV is inadequate, such as northern Nigeria, (*11*,*36*) where 24 emergence events were observed (*11*,*37*). Favorable conditions for VDPV emergence existed throughout much of DRC.

More than half of the VDPVs identified were associated with cases from Katanga and were isolated during 2005, 2008, 2009, and 2011. WPV1 circulated in Katanga in 2010 and 2011 in districts other than those with circulating VDPV. Social mobilization efforts to increase vaccination acceptance are ongoing in Katanga because of a relatively high frequency of parents who refuse to have their children vaccinated, some for religious reasons (*38*).

The relative risks for paralysis from WPV1 and VDPV2 seem to be driven primarily by the particular serotype; the disease-to-infection ratio is higher for type 1 than for type 2. In a study of acute flaccid paralysis patients in Nigeria, clinical characteristics of cases associated with VDPV infection were similar to those associated with cases of WPV1 infection (*11*). Similar studies have not been performed for DRC.

Vaccination activities conducted in response to the outbreaks in 2008 and 2009 probably contributed to the

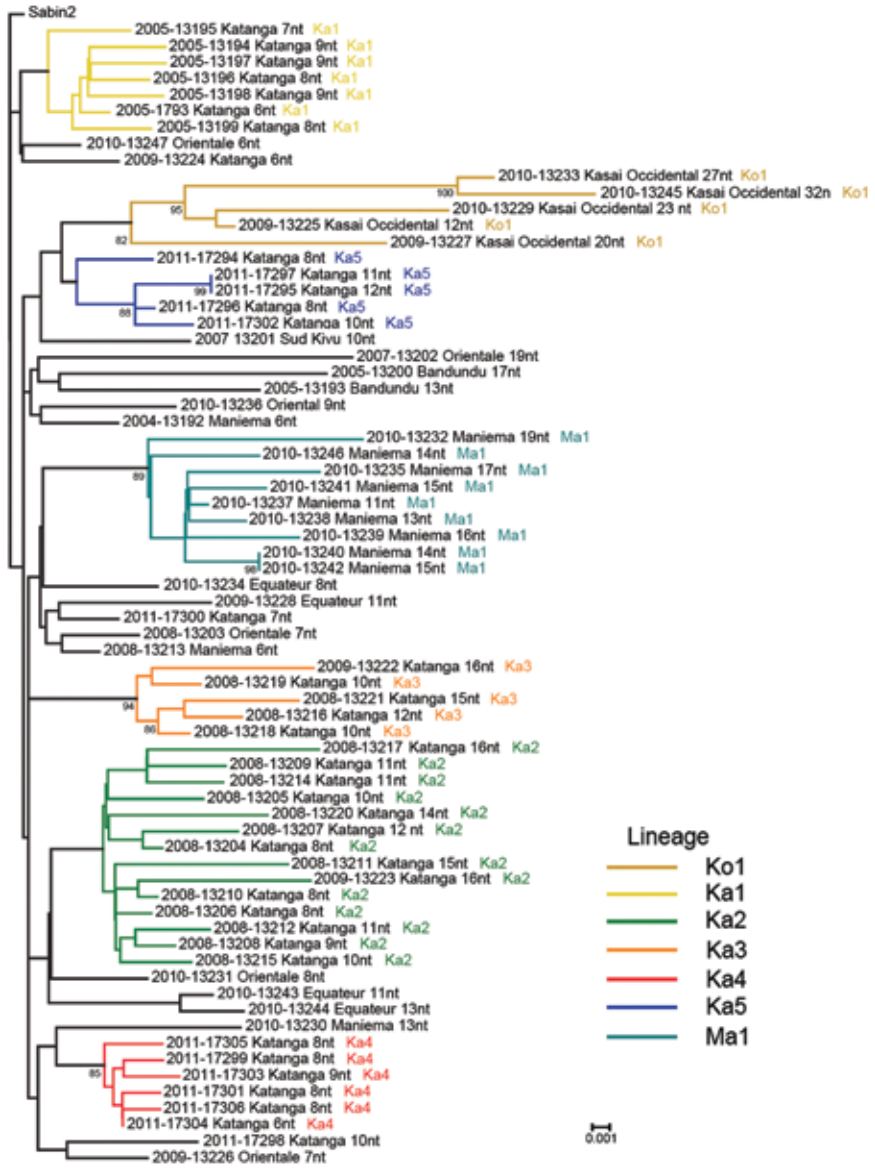

Figure 3. Viral protein phylogenetic relationships among vaccine-derived poliviruses isolated from patients with acute flaccid paralysis, Democratic Republic of Congo, 2004–2011. The tree was rooted to the Sabin type 2 poliovirus sequence. The year of onset of paralysis is indicated at the beginning of each virus name, followed by a 5-digit identifier and the province of the case-patient. The numbers of nucleotide differences from the Sabin 2 prototype viral protein 1 sequence are indicated, followed by the name of the lineage. Bootstrap values >80 are indicated on the tree. Scale bar indicates nucleotide substitutions per site.

disruption of VDPV and WPV transmission in 2010. In response to the VDPV2 outbreak, mass vaccination campaigns were performed in areas of Maniema, Kasai Occidental, and Katanga. Trivalent OPV was used in these campaigns. Monovalent OPV1 and bivalent OPV were used to control circulating WPV1. After circulation of lineages Ka1–Ka3 had stopped, circulating VDPV was again detected in Katanga in late 2011 (Ka4 and Ka5). As in 2008, simultaneous emergence of >1 VDPV lineage occurred in Katanga. Several VDPVs were detected in 2012 in Katanga; their characterization is ongoing (Qi Chen et al., unpub. data).

Events that occurred early in the circulating VDPV emergence pathway were observed for some lineages. OPV strains that differ from Sabin strains by 1–5 nt are being routinely identified by the sensitive VDPV screening assay in use in Global Polio Laboratory Network laboratories, and these minimally drifted Sabin-related viruses can occasionally be linked to circulating VDPVs. Figure 3 includes several isolates that differ from the Sabin 2 prototype VP1 sequence by 6 nt. A Katanga isolate that differed from the Sabin strain by 3 nt was genetically linked to a Katanga VDPV (13224) that differed from the Sabin strain by 6 nt (data not shown).

Before use of the sensitive VDPV screening assay was implemented, events that occurred early in the circulating VDPV emergence pathway were not observed. In the circulating VDPV type 1 outbreak in Hispaniola, early events were missed because of delayed recognition of the outbreak. The study of early events in circulating VDPV emergence is

yielding valuable information for use in planning the endgame strategy for polio eradication. It now seems necessary to synchronize the phase-out of OPV to minimize the emergence of circulating VDPVs, particularly circulating VDPV2. Emergence of VDPVs from the Sabin OPV2 strain has been much more frequent than emergence of VDPVs from OPV1 and OPV3. Discontinuation of the type 2 component of OPV is being considered as a strategy for reducing the frequency of circulating VDPV2 emergence (*12*).

In this study, each DRC viral lineage was found to be restricted to a limited geographic region. This finding is consistent with the relative lack of long-range transportation networks across DRC and the low rate of routine vaccination coverage throughout DRC, which results in gaps in immunity to type 2 PV. Without an extensively developed highway system, individual geographic regions tend to be isolated and human population movement is restricted. With limited movement of people across long distances, virus transmission is restricted. The situation in DRC contrasts with that in northern Nigeria, where large-scale population movement occurs between the west and east across the northern states. Wild and circulating VDPV lineages spread widely throughout the northern states of Nigeria (*11*).

The circulating VDPVs detected in DRC had recovered the 2 most important biological properties of WPVs, namely the capacity to cause paralytic disease in humans and the capacity for continuous person-to-person transmission. The origin of circulating VDPV2 in DRC was probably the result of low population immunity to PV because of a combination of low vaccination coverage in some communities (*39*) and the prior elimination of the indigenous WPV of the same serotype (*20*). Because WPV2 has not circulated anywhere globally since 1999, immunity to PV type 2 is not being stimulated through virus circulation. Routine vaccination coverage is low in many regions of DRC; estimated OPV3 coverage was 73% (*40*). Additional factors that probably facilitated circulation of VDPVs in some communities were poor hygiene, inadequate sanitation, and tropical climate. Because similar conditions exist elsewhere, ongoing high-quality surveillance will be essential for eliminating polio in Africa. The need for vigilance was confirmed by the detection of circulating VDPV2 and circulating VDPV3 in Ethiopia (Gumede et al., unpub. data), circulating VDPV2 in Chad, and circulating VDPV2 in Somalia (*20*). The occurrence of WPV outbreaks in DRC emphasizes the need to maintain high vaccine coverage and acute flaccid paralysis surveillance to minimize the risk for emergence of VDPVs or circulation of imported WPVs.

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Coccidioidomycosis-associated Hospitalizations, California, USA, 2000–2011

Gail Sondermeyer, Lauren Lee, Debra Gilliss, Farzaneh Tabnak, and Duc Vugia

In the past decade, state-specific increases in the number of reported cases of coccidioidomycosis have been observed in areas of California and Arizona where the disease is endemic. Although most coccidioidomycosis is asymptomatic or mild, infection can lead to severe pulmonary or disseminated disease requiring hospitalization and costly disease management. To determine the epidemiology of cases and toll of coccidioidomycosis-associated hospitalizations in California, we reviewed hospital discharge data for 2000–2011. During this period, there were 25,217 coccidioidomycosis-associated hospitalizations for 15,747 patients and >$2 billion US in total hospital charges. Annual initial hospitalization rates increased from 2.3 initial hospitalizations/100,000 population in 2000 to 5.0 initial hospitalizations/100,000 population in 2011. During this period, initial hospitalization rates were higher for men than women, African Americans and Hispanics than Whites, and older persons than younger persons. In California, the increasing health- and cost-related effects of coccidioidomycosis-associated hospitalizations are a major public health challenge.

Coccidioidomycosis, also known as Valley fever, is a reemerging infectious disease caused by inhalation of *Coccidioides* fungi spores, which reside in the soil of regions where coccidioidomycosis is endemic, including the southwestern United States (*1*–*5*). California and Arizona have the highest rates of reported coccidioidomycosis cases in the United States (*4*,*6*). In California, the pathogen is most common in the San Joaquin Valley, and compared with other Californians, residents of this region are at increased risk for infection (*6*,*7*). Although most coccidioidomycosis cases are asymptomatic, symptomatic disease will develop in ≈40% of patients and can range from self-limited influenza-like illness to disseminated disease and chronic meningitis (*7*,*8*). Symptomatic disease can require expensive and aggressive treatments, prolonged absence from work or school, multiple hospitalizations, and years of monitoring (*9*,*10*). Populations at particular risk for severe disease include African Americans, immunocompromised persons, and persons ≥65 years of age (*2*,*11*).

Over the past decade, increases in the number of reported cases of coccidioidomycosis have been documented from Arizona and California (*6*,*12*–*16*). In California, the rates of reported cases increased >6-fold from 2000 to 2011 (2.4 to 14.4 cases/100,000 population, respectively) (*13*,*15*). Cases among prisoners in California also increased during this time, making prisoners a population of concern (*17*,*18*). To determine the epidemiology, extent, and effect of the disease in California, we reviewed coccidioidomycosis-associated hospitalizations in the state for 2000–2011.

Methods

Data Source

We used the California Patient Discharge Data Set, an administrative database developed by the Office of Statewide Health Planning and Development, to review hospitalization data for 2000–2011 (*19*). The database contains inpatient (defined as a person formally admitted to the hospital with the expectation of remaining overnight or longer) information reported by nonfederal hospitals in California (*20*). These data include diagnosis and procedure codes, demographic characteristics, hospital admission and discharge dates, source of admission, and hospital charges. Records for coccidioidomycosis-associated hospitalizations were identified as records with an admission date during January 1, 2000–December 31, 2011, and 1 of the following primary or secondary International Classification of Diseases, 9th revision (ICD-9), codes for coccidioidomycosis: 114.0 primary pulmonary, 114.1 primary extrapulmonary, 114.2 meningitis, 114.3 other forms of progressive, 114.4 chronic pulmonary, 114.5 pulmonary unspecified, and 114.9 unspecified.

Hospitalization records were matched by patients' social security numbers, dates of birth, sex, race/ethnicity, and county of residence by using probabilistic record

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linkage methods as described (*21*,*22*). Matching of records by patient enabled the classification of all hospitalization records into initial and subsequent hospitalizations for each patient. Records missing the social security number or other data elements were included in the record linkage and subsequent analyses.

Hospitalization Rates

Numbers of coccidioidomycosis-associated hospitalizations were calculated for each year during 2000–2011, by initial and subsequent hospitalization, and by type of coccidioidomycosis. Hospitalization rates were calculated per 100,000 population by using population estimates from the California Department of Finance that were based on the 2010 census (*23*,*24*).

Demographic Characteristics

Subanalyses were performed for patient initial hospitalizations by sex, age group, race/ethnicity, and county and region of patient residence. Two regions of California were defined for this study: areas where coccidioidomycosis is endemic (hereafter referred to as the endemic region) and areas where coccidioidomycosis is less endemic (hereafter referred to as the less endemic region). The endemic region was defined as the 6 counties that had annual case rates consistently higher than those for the state during 2001–2011: Fresno, Kings, Kern, Madera, San Luis Obispo, and Tulare Counties (*6*,*12*–*14*). The less endemic region was defined as all other California counties. Descriptive statistics were also calculated for hospital admissions from prisons or jails.

Concurrent Conditions

We reviewed diagnosis codes for the initial hospitalization records to identify patients with the following concurrent conditions: immunocompromised state as defined by the Agency for Healthcare Research and Quality (*25*), HIV and AIDS (ICD-9 codes 042, V08), diabetes (ICD-9 code 250), and pregnant state (ICD-9 codes V22, V23, 630–679). The estimated percentage of the California population with these conditions was then provided as a reference. The estimated percentage of the California population with HIV infection or AIDS was determined by using data from the California Department of Public Health HIV/AIDS Surveillance Statistical Reports for 2004–2011, and the estimated percentages of the California population with diabetes and with pregnancy were determined by averaging data from the California Health Interview Survey (CHIS) for 2003, 2005, 2007, and 2009 (*26*,*27*). For diabetes, both the percentage in our study and the estimated population average were age-adjusted to the 2000 standard US population. We were unable to determine the prevalence of non-HIV immunocompromised state in the population.

Length of Stay and Hospital Charges

Length of stay was calculated for all initial and subsequent hospitalizations and was defined as the number of days from admission to discharge for each hospitalization. Total length of stay per person was defined as the sum of the lengths of stay for an individual patient's initial and subsequent hospitalizations during the study period.

Total hospital charge per patient was calculated by adding the total charges for all hospitalizations during 2000–2011. Total hospital charge per day was determined by dividing the total charge for each hospitalization by the length of stay for that record. The sum of charges for all hospitalizations was stratified by the 10 expected source of payment categories defined by the Office of Statewide Health Planning and Development (*19*). Hospital charges for Medi-Cal, Medicare, county indigent programs, and other government were combined to estimate the total charge to government payers. The annual sums of charges were adjusted for inflation and standardized to 2011 by using the US Department of Labor Consumer Price Index and were used to assess changes in total charges over time (*28*). All charge figures were calculated in US dollars. When a total charge was indicated as invalid, unknown, or no charge, the records were excluded from analyses. Approximately 8% of hospitalization records had invalid, unknown, or no charge indicated.

Statistical Analysis

For all, initial, and subsequent hospitalizations and for all primary pulmonary, other forms of progressive, and meningeal coccidioidomycosis hospitalizations, *z* tests were used to compare rates for 2011 with those for 2000. In addition, bivariate relative risks were calculated for the effects of year (2000–2011), sex (female, male), age group (0–19, 20–39, 40–59, and ≥60 years), and race/ethnicity (White, African American, Hispanic, Other) on patient initial hospitalization. Factors with significant bivariate relative risks were then included in a multivariate negative binomial regression model, which was used to test for statistical significance of the trend in initial hospitalization statewide during 2000–2011. Negative binomial regression models controlling for sex, age group, and race/ethnicity were also used to test for statistical significance of the trends in initial hospitalization in the endemic and less endemic regions. Statistical significance was defined as a p value of ≤0.05. SAS 9.2 software (SAS Institute Inc., Cary, NC, USA) was used for analyses.

Results

Hospitalizations and Coccidioidomycosis Diagnoses

During 2000–2011, there were 25,217 coccidioidomycosis-associated hospitalizations in California; hospitalizations increased from 1,074 in 2000 to 3,197

in 2011 (Table 1). The hospitalization rate per 100,000 population in 2011 was 8.6, a significant increase from the 2000 rate of 3.2/100,000 population (p<0.0001). Of the 25,217 hospitalizations, 15,747 (62%) were initial hospitalizations and 9,470 (38%) were subsequent hospitalizations. Of the initial hospitalizations, 9,568 (61%) were for a primary diagnosis of coccidioidomycosis. The initial hospitalization rate per 100,000 population in 2011 was 5.0, a 2-fold increase from the 2000 rate of 2.3/100,000 population (p<0.0001), and the rate of subsequent hospitalizations in 2011 was 3.6/100,000 population, a >4-fold increase from the 2000 rate of 0.8/100,000 population (p<0.0001).

For all hospitalizations, the most common types of coccidioidomycosis diagnoses were for primary pulmonary (48%), other forms of progressive (18%), and coccidioidal meningitis (13%) (Table 1). The rate of any hospitalization for primary pulmonary coccidioidomycosis was ≈4-fold higher in 2011 than in 2000 (4.3 vs. 1.1 hospitalizations/100,000 population), and the rate of hospitalization for other forms of progressive disease and meningitis was ≈2-fold higher in 2011 than in 2000 (p<0.0001 for all).

Of 15,747 patients initially hospitalized for coccidioidomycosis, 3,824 (24%) were readmitted to a hospital during the study period: 2,004 (52%) were readmitted once, 746 (20%) were readmitted twice, and 1,074 (28%) were readmitted ≥3 times. The median time from initial hospitalization discharge to first readmission was 47 days (range 0–4,252 days). Of those readmitted, 3,006 (79%) were readmitted within 1 year after the initial hospitalization discharge.

A total of 1,220 (8%) patients hospitalized for coccidioidomycosis died during an initial or subsequent hospitalization. For these patients, there was a diagnosis during the final hospitalization of primary pulmonary coccidioidomycosis for 628 (51%), other forms of progressive disease for 309 (25%), and coccidioidal meningitis for 196 (16%).

Hospitalizations by Region

During the study period, hospitalized patients were residents of 56 of the 58 California counties. Approximately 50% of patients initially hospitalized for coccidioidomycosis resided in 1 of the 6 endemic region counties. The rate of initial hospitalizations in the endemic region increased nearly 3-fold from 2000 (12.0 initial hospitalizations/100,000 population) through 2011 (34.6 initial hospitalizations/100,000); in the less endemic region, the rate increased 1.6-fold from 2000 (1.6 initial hospitalizations/100,000 population) through 2011 (2.5 initial hospitalizations/100,000 population) (Table 2). In the endemic region, the highest annual hospitalization rates were consistently observed in Kern and Kings Counties.

Hospitalization Trends

The annual number of hospitalizations increased steadily during 2000–2006, followed by a slight decrease through 2008 and then a steady increase through 2011 (Figure 1). Using multivariate analysis, we adjusted for sex, age group, and race/ethnicity and determined that the increasing trend in initial hospitalizations during 2000–2011 was significant (p<0.0001). Over this period, there was also an overall increase in the annual rates of initial hospitalizations in the endemic and less endemic regions; however, rates in the endemic region were 7–14 times higher than those in the less endemic region (Figure 2). When controlled for sex, age group, and race/ethnicity, trends in initial hospitalizations in the 2 regions increased significantly (p<0.0001) during 2000–2011.

Demographic Characteristics

During 2000–2011, the overall initial hospitalization rate increased 2-fold; when the analysis was stratified by sex, age group, and race/ethnicity, a similar increase was observed within most strata (Table 3). Most (69%) initial hospitalizations were for men; the annual initial hospitalization rate for men (6.7/100,000 population in 2011) was

Table 1. Coccidioidomycosis-associated hospitalizations, California, 2000–2011

Variable	No. (%), 2000–2011	No. (rate/100,000 population) 2000	2011
Hospitalizations			
All	25,217 (100)	1,074 (3.2)	3,197 (8.6)*
Initial	15,747 (62.4)	798 (2.3)	1,851 (5.0)*
Subsequent	9,470 (37.6)	276 (0.8)	1,346 (3.6)*
Type of coccidioidomycosis†			
Primary pulmonary	12,041 (47.7)	372 (1.1)	1,609 (4.3)*
Other forms of progressive	4,539 (18.0)	224 (0.7)	559 (1.5)*
Meningitis	3,208 (12.7)	195 (0.6)	384 (1.0)*
Pulmonary, unspecified	2,657 (10.5)	124 (0.4)	335 (0.9)
Unspecified	2,372 (9.4)	105 (0.3)	320 (0.9)
Chronic pulmonary	1,159 (4.6)	80 (0.2)	111 (0.3)
Primary extrapulmonary	163 (0.6)	5 (<0.1)	14 (<0.1)

*All hospitalization rates for 2011 were significantly greater than those for 2000 (p<0.0001, z tests).
†Numbers, percentages, and rates of all hospitalizations (n = 25,217). Multiple coccidioidomycosis diagnosis codes may be indicated for a single hospitalization.

consistently more than twice that for women (3.2/100,000 population in 2011). In most years, the annual initial hospitalization rates increased steadily by advancing age group and then decreased slightly for patients ≥80 years of age; the highest 2011 initial hospitalization rate (8.5/100,000 population) was for persons 70–79 years of age. For race/ethnicity, the annual initial hospitalization rates were highest for African Americans (8.9/100,000 population in 2011) and lowest for Asian/Pacific Islanders (2.0/100,000 population in 2011).

Multivariate negative binomial regression analysis showed that male sex, older age group, and African American and Hispanic race/ethnicities were significantly associated with an increased risk for initial hospitalization ($p<0.0001$ for all) (Table 4). This increased risk was 2.48 times higher for men than women. The risk for initial hospitalization for African Americans and Hispanics was 2.09 and 1.31 times higher, respectively, than that for Whites. The risks for initial hospitalization for persons 20–39, 40–59, and ≥60 years of age were 4.22, 7.73, and 9.50 times higher, respectively, than that for persons 0–19 years of age (Table 4).

A total of 1,374 (8.7%) patients initially hospitalized with coccidioidomycosis were admitted from a prison or jail. Of those, 1,103 (80%) were admitted from prisons or jails in the endemic region of California. Thirty-eight percent of hospitalized persons from Kings County were initially admitted from prison or jail, compared with 25% of persons from Fresno County and 10% from Kern County. The median age of persons admitted from prison or jail was 42 years (range 18–88), and 99.5% of these patients were men. Of the 1,374 patients initially admitted to the hospital from prison or jail, 342 (25%) were White, 379 (28%) were African American, and 419 (30%) were Hispanic. The number of initial hospital admissions for persons in prison or jail increased from 28 in 2000 to 201 in 2011. When initial hospitalizations for persons in prison or jail were excluded from the multivariate analysis, the risk for initial hospitalization for men, persons in older age groups, and persons of African American or Hispanic race/ethnicity remained significantly higher than the risk for the reference group ($p<0.05$ for all) (data not shown).

Records for 33% (5,176) of the coccidioidomycosis-related initial hospitalizations had a diagnosis code indicating a concurrent condition that could increase the risk for infection or severe disease. Of those, 34% (1,760) had diagnosis codes for immunocompromising conditions. Approximately 2.8% (439) of initial hospitalizations had a diagnosis code of HIV infection or AIDS, compared with an estimated 0.3% of the California population. Approximately 25% (3,786) of adults initially hospitalized for coccidioidomycosis had a diagnosis of diabetes. When our data and the CHIS data were age-adjusted to the 2000 US standard population, 22% had a diagnosis of diabetes, compared with an age-adjusted 7.7% of the general California population. Twelve percent (185) of women 18–45 years of age had a diagnosis code indicating pregnancy, compared with the state average of 4% from CHIS.

Length of Stay and Hospital Charges

During 2000–2011, the median length of stay per hospitalization was 6 days; this length of stay did not vary substantially over time. For ≈52% of those hospitalized, the total per person length of stay was >1 week. The median charge per day was ≈$6,800 US, and the median total hospital charge per patient was US $55,062 (range ≈$1,000 to >$6 million).

During 2000–2011, the total charges for all coccidioidomycosis-associated hospitalizations in California was US $2.2 billion, and the average annual total was US $186 million (Table 5). After we adjusted for inflation, the annual total charges increased from US $73 million in 2000 to US $308 million in 2011. For the expected source of payment category, private coverage had the highest total hospital charges during the study period (total US $713 million; average annual total US $59 million). Government payers (defined as Medi-Cal, Medicare, other government, and county indigent payers) were the expected source of payment for 62% of charges. For all government payers combined, the total (US $1.4 billion) and average annual

Table 2. Coccidioidomycosis-associated initial hospitalizations by patient residence, California, 2000–2011*

Patient residence†	No. (%), 2000–2011, N = 15,747	No. (rate/100,000 population) 2000	2011
Endemic region, county	7,683 (48.8)	281 (12.0)	990 (34.6)
Fresno	1,450 (9.2)	30 (3.7)	171 (18.1)
Kern	4,016 (25.5)	155 (23.3)	544 (61.9)
Kings	760 (4.8)	20 (15.4)	99 (63.5)
Madera	125 (0.8)	5 (4.0)	20 (12.8)
San Luis Obispo	398 (2.5)	23 (9.3)	49 (17.9)
Tulare	934 (5.9)	48 (13.0)	107 (23.6)
Less endemic region	8,064 (51.2)	517 (1.6)	861 (2.5)

*For this study, 6 California counties where coccidioidomycosis is endemic were defined as the endemic region, and all other counties, where coccidioidomycosis is less endemic, were defined as the less endemic region.
†For patients admitted from prison or jail, patient residence was based on the location of the prison or jail.

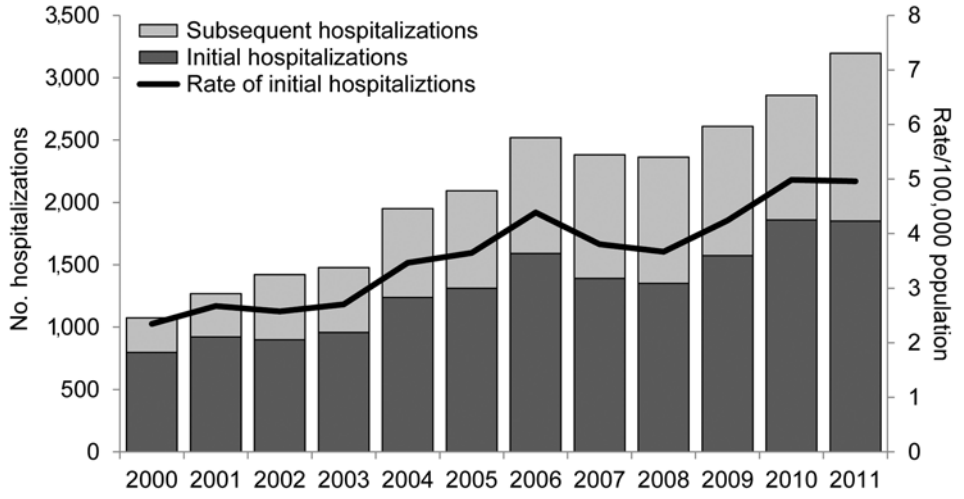

Figure 1. Numbers and annual rates of initial and subsequent coccidioidomycosis-associated hospitalizations (N = 25,217) by year of admission, California, 2000–2011.

total (US $115 million) of hospital charges were each nearly twice those for private coverage (Table 5).

Discussion

The number of initial and subsequent coccidioidomycosis-associated hospitalizations in California increased substantially during 2000–2011, totaling >$2 billion US in hospital charges, most of which was covered by government funding. Rates for initial hospitalizations were substantially higher for men than women, for African Americans and Hispanics than Whites, for persons of older age than younger age, and for persons residing in endemic region counties than in less endemic region counties.

Our study documents a large financial cost related to coccidioidomycosis-associated hospitalizations in California, yet our estimates do not include indirect costs linked to the hospitalizations (e.g., costs related to child care, missed days of work, and follow-up outpatient care). These costs are likely substantial because >50% of patients were hospitalized for >1 week. In addition, hospitalization charges are only a portion of the total financial cost of coccidioidomycosis because most patients do not require hospitalization but may accrue substantial costs during outpatient care (*9*).

The increases in coccidioidomycosis-associated hospitalizations in California were observed in the endemic and less endemic regions. Nearly 50% of initial hospitalizations were for persons residing in the endemic region counties, even though only 7% of the California population lived in these counties during 2000–2011. The increase in hospitalizations is consistent with the dramatic increase in the number of reported coccidioidomycosis cases in California, yet the reasons for the increase are unclear (*6*,*12*–*15*). Contributing factors may include changes in climate and rainfall patterns, leading to proliferation of *C. immitis* fungi in the

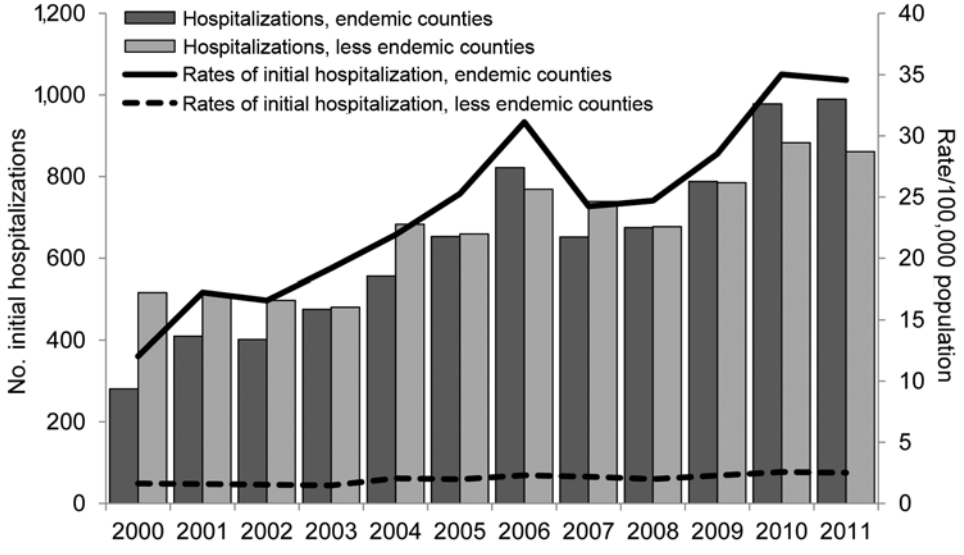

Figure 2. Numbers and annual rates of initial coccidioidomycosis-associated hospitalizations (N = 15,747) in endemic and less endemic regions of California by year of admission, 2000–2011. For this study, 6 California counties (Fresno, Kings, Kern, Madera, San Luis Obispo, and Tulare) where coccidioidomycosis is endemic were defined as the endemic region, and all other counties, where coccidioidomycosis is less endemic, were defined as the less endemic region.

Table 3. Coccidioidomycosis-associated initial hospitalizations by patient demographic characteristics, California, 2000–2011

Characteristic	No. (%), 2000–2011, N = 15,747	No. (rate/100,000 population)	
		2000, n = 798 (2.3)	2011, n = 1,851 (5.0)
Sex			
F	4,870 (30.9)	256 (1.5)	607 (3.2)
M	10,876 (69.1)	542 (3.2)	1,244 (6.7)
Age, y			
0–9	287 (1.8)	8 (0.2)	38 (0.8)
10–19	680 (4.3)	25 (0.5)	91 (1.7)
20–29	1,630 (10.4)	64 (1.3)	210 (3.7)
30–39	2,476 (15.7)	147 (2.7)	280 (5.4)
40–49	3,312 (21.0)	162 (3.2)	361 (7.0)
50–59	3,190 (20.3)	181 (5.2)	399 (8.3)
60–69	2,032 (12.9)	99 (4.7)	235 (7.2)
70–79	1,375 (8.7)	78 (4.6)	150 (8.5)
≥80	765 (4.9)	34 (3.7)	87 (7.2)
Race/ethnicity			
White	6,612 (42.7)	381 (2.4)	697 (4.8)
African American	1,886 (12.2)	106 (4.8)	190 (8.9)
Hispanic	5,469 (35.3)	233 (2.1)	747 (5.2)
Native American/Alaska Native	66 (0.4)	3 (1.7)	10 (6.5)
Asian/Pacific Islander	1,033 (6.7)	52 (1.3)	104 (2.0)
Other	425 (2.7)	12 (1.9)	66 (7.2)

soil; soil-disturbing construction activities; an increase in susceptible persons moving to disease-endemic areas; and heightened awareness and diagnosis (*1*,*2*,*5*,*16*,*29*).

The high initial hospitalization rate for men in our study is consistent with findings from previous research. This finding may reflect the higher risk for men than women of developing primary pulmonary and extrapulmonary disease once infected, and it may reflect an increased risk for infection among men because of dust exposure in male-dominated occupations, such as construction and farm work (*7*,*8*,*30*,*31*). The high initial hospitalization rates observed for African American and Hispanic residents may be associated with the well-known increased risk for disseminated disease in African Americans and with a reported increased risk for symptomatic disease for both of these racial/ethnic groups in California (*6*,*7*,*32*). Hector et al. (*6*) reported that of the coccidioidomycosis cases reported in California during 2001–2009 with complete race/ethnicity data, a higher proportion were among persons of African American and Hispanic race/ethnicity than would be expected on the basis of the proportion of these racial/ethnic groups in the population. A contributing factor to this finding may be the large populations of Hispanics living and working in the endemic region counties of California. However, the principle causes of the disproportionate race/ethnicity-associated risk for coccidioidomycosis is not well understood and may be attributable to variations in genetic susceptibility (*6*,*7*,*32*).

The percentages of coccidioidomycosis-infected persons hospitalized with HIV infection or AIDS, diabetes, and pregnancy were greater than estimated population percentages, and these conditions are known to increase the risk for developing severe pulmonary or disseminated coccidioidomycosis (*7*,*33*). It is possible, however, that these concurrent conditions put persons at risk for hospitalization in general and that some hospitalizations were primarily for the concurrent condition rather than coccidioidomycosis. An increased risk for severe disease associated with concurrent conditions may also explain the higher rate of initial hospitalization that we observed for persons in older age groups; it has been reported that decreasing immunity in the elderly is correlated with the presence of concurrent conditions (*33*,*34*). In addition, the decline in immunity in elderly persons particularly affects cell-mediated immunity, which is vital for protection against coccidioidomycosis (*33*,*35*).

Almost 9% of patients initially hospitalized for coccidioidomycosis were admitted from prison or jail. A high proportion of these patients were nonwhite men, a finding that reflects the general California inmate population

Table 4. Risk for coccidioidomycosis-associated initial hospitalization, by various demographic characteristics, California, 2000–2011*

Characteristic	Multivariate RR (95% CI)†
Year	1.06 (1.06–1.07)
Sex	
F	Referent
M	2.48 (2.33–2.63)
Age, y	
0–19	Referent
20–39	4.22 (3.82–4.66)
40–59	7.73 (7.01–8.52)
≥60	9.50 (8.59–10.50)
Race/ethnicity	
White	Referent
African American	2.09 (1.92–2.28)
Hispanic	1.31 (1.21–1.41)
Other‡	0.83 (0.76–0.90)

*N = 15,747. RR, relative risk.
†RRs were calculated by using multivariate negative binomial regression. All p values were <0.0001.
‡Includes persons of Asian/Pacific Islander, Native American/Alaska Native, and other race/ethnicity.

Table 5. Total and average annual charges, by expected source of payment category, for coccidioidomycosis-associated hospitalizations, California, 2000–2011*

Payment category	Total charges, US $ (%)	Average annual charges, US $
Private coverage	713,390,109 (32)	59,449,176
Government	1,388,671,670 (62)	115,722,639
Medi-Cal	595,837,721 (27)	49,653,143
Medicare	567,965,499 (25)	47,330,458
Other government	161,878,874 (7)	13,489,906
County indigent programs	62,989,577 (3)	5,249,131
Self-pay	92,892,777 (4)	7,741,065
Workers compensation	18,209,024 (1)	1,517,419
Other payer	11,775,902 (1)	981,325
Other indigent	7,553,126 (<1)	629,427
Invalid/unknown	593,365 (<1)	118,673
Total charges	2,233,085,973 (100)	186,159,724

*Unknown, invalid, and missing charges were excluded from this analysis. Charity care charges coded as $1 were also excluded. Approximately 8% of hospitalizations were missing total charge data. These charges are not adjusted for inflation.

(*36*). Of the 33 California correctional and rehabilitation facilities for adults, 11 are located in the endemic region, and most patients in this study who were hospitalized from prison or jail were admitted from facilities in the endemic region. However, many prisoners in facilities in the endemic region may have resided in or been transferred from counties in the less endemic region, and immunity to coccidioidomycosis would have been less likely to have developed in persons from the less endemic region. Despite the demographic differences between patients admitted to the hospital from prison or jail and those admitted from the general population, the results of the multivariate analyses did not change substantially when data for prison- or jail-admitted hospitalizations were included.

There were several limitations in this study. Some hospitalizations with a secondary diagnosis code for coccidioidomycosis could have been for a condition unrelated to coccidioidomycosis, and inclusion of such hospitalizations in the analyses could have led to an overestimation of the incidence of disease. Some readmissions (e.g., some occurring >1 year after discharge for the initial hospitalization) might also have been for an unrelated medical issue. However, most of the initial hospitalizations had a primary diagnosis of coccidioidomycosis, and most first readmissions occurred <1 year after initial discharge. Some hospitalizations in this study may have also been misclassified as initial hospitalizations because data were not reviewed to identify coccidioidomycosis-associated hospitalizations that occurred before January 1, 2000. Such a misclassification would have led to an overestimation of the number of initial hospitalizations occurring in the earlier years more than in the later years; if that was the case, it would indicate that the increasing trend found in initial hospitalizations may be even greater. The estimates of total charges likely underestimated the financial cost of coccidioidomycosis-associated hospitalizations because charge data were missing for >8% of the hospitalizations. In addition, the California Patient Discharge Data Set did not collect data from federal hospitals in California, a fact that would lead to an underestimation of hospitalization and hospital charge figures.

The increasing health and financial toll of coccidioidomycosis-associated hospitalizations in California are a major public health challenge. Efforts are needed to reduce the incidence of disease, yet options for the prevention of coccidioidomycosis are limited. Although a vaccine is not currently available, vaccine research is under way (*37*). Early diagnosis, close follow up, and appropriate treatment of patients at risk for severe or disseminated disease may decrease the number of long-term illnesses and deaths. Thus, efforts should be made to increase disease awareness and promote early recognition among health care providers and the public. In addition, prevention messages on how to minimize or avoid breathing in dusty air should be communicated more widely to persons living in or traveling to areas where *Coccidioides* fungi are endemic, particularly to persons at risk for severe disease and hospitalization.

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Immunogenic *Mycobacterium africanum* Strains Associated with Ongoing Transmission in The Gambia

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In West Africa, *Mycobacterium tuberculosis* strains cocirculate with *M. africanum*, and both pathogens cause pulmonary tuberculosis in humans. Given recent findings that *M. tuberculosis* T-cell epitopes are hyperconserved, we hypothesized that more immunogenic strains have increased capacity to spread within the human host population. We investigated the relationship between the composition of the mycobacterial population in The Gambia, as measured by spoligotype analysis, and the immunogenicity of these strains as measured by purified protein derivative–induced interferon-γ release in ELISPOT assays of peripheral blood mononuclear cells. We found a positive correlation between strains with superior spreading capacity and their relative immunogenicity. Although our observation is true for *M. tuberculosis* and *M. africanum* strains, the association was especially pronounced in 1 *M. africanum* sublineage, characterized by spoligotype shared international type 181, which is responsible for 20% of all tuberculosis cases in the region and therefore poses a major public health threat in The Gambia.

Tuberculosis (TB), caused by bacterial pathogens of the *Mycobacterium tuberculosis* complex (MTBC), is a major global health problem. Sub-Saharan Africa has the highest rate of TB per capita and the lowest case detection rate; although TB incidence is decreasing globally, incidence rates are increasing in most countries in the West Africa region (*1*). Moreover, almost half of all TB cases in West Africa are caused by infection with an unusual member of the MTBC, *M. africanum*, a lineage found exclusively in this region. Although *M. africanum* was initially described in Senegal in 1968 (*2*), and despite its importance and high prevalence in this region, relatively little is known about the bacterium (*3*). In general, *M. africanum* can be divided into 2 lineages: Afri_1, by SpolDB4 definition (*4*), corresponding to the green lineage 6 (*5*), which has the highest prevalence in Senegal, Mali, The Gambia, Guinea-Bissau, and Sierra Leone (*3*); and Afri_2 (*4*), corresponding to the brown lineage 5 (*5*), which is mainly found in the eastern part of West Africa, in countries such as Côte d'Ivoire, Ghana, Benin, Nigeria, and Cameroon (*3*).

Although transmission of *M. africanum* from host to host is a crucial element of the spread of the disease, the underlying biological mechanisms triggering transmission are elusive. We assessed transmission dynamics and interaction between the 2 mycobacterial populations in The Gambia, a country in western West Africa, and compared the local situation with previously published data from Guinea-Bissau, another country within the region (*6*). In particular, considering a recent publication suggesting that conserved mycobacterial T-cell epitopes may play a role in the transmission of the mycobacteria within the host population (*7*), we investigated whether differences in immunogenicity between *M. tuberculosis* and *M. africanum* strains (especially of the predominant Euro-American [EA] and Afri_1 lineages) could predict the success of certain sublineages to transmit and establish themselves within the human host population.

Materials and Methods

Study Population and Sample Collection

Data for our study came from an ongoing TB case–contact study at the Medical Research Council in The Gambia;

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TB case-patients were recruited for that study during June 20, 2002–December 21, 2009. Consecutive patients were included after written informed consent if they were ≥15 years old, resided in the study area (Greater Banjul area), and produced 2 sputum samples that were positive for acid-fast bacilli by Ziehl-Neelsen staining.

Spoligotyping and Analysis

Genomic DNA was purified from the collected sputum samples by using the cetyl trimethyl ammonium bromide and chloroform method, as described (*8*). Spoligotyping was performed by using commercially available membranes (Ocimum Biolsolutions, Hyderabad, India), according to standardized protocols (*9*). Spoligotype patterns with ambiguous signature were confirmed by using long-sequence polymorphism PCR.

In addition to analyzing the samples collected in The Gambia, we reanalyzed spoligotypes from published studies from Guinea-Bissau (*6*). The shared international type (SIT) number was assigned by using the SITVIT database on the Institute Pasteur de Guadeloupe website (www.pasteur-guadeloupe.fr/tb/bd_myco.html). Lineages of spoligotypes were assigned according to SpolDB4 classification by using the TB Lineage online platform (http://tbinsight.cs.rpi.edu/about_tb_lineage.html) (*10*). Spoligotype data were further analyzed by using spolTools (www.emi.unsw.edu.au/spolTools) (*11*), which provides online tools for the construction of Spoligoforests (*12*) and to Detect Emerging Strains of Tuberculosis by Using Spoligotyping (DESTUS) (*13*) from spoligotype data. As recommended by the provider, the Spoligoforests and DESTUS programs were run with the default settings.

To analyze temporal clustering of spoligotypes from The Gambia, we used SaTScan version 9.1.1 software (www.satscan.org) (*14*); we conducted a purely temporal analysis for high rates of clustering in a discrete Poisson model. Hospital admission dates for each patient were used as input dates for each spoligotype, and the resolution of the analysis was set to days.

PPD-ELISPOT

Purified protein derivative (PPD) ELISPOT assays were performed as described (*15*) on a subset of 372 study samples. Quantitative results were expressed as the number of spot-forming units (sfu) that produce interferon-γ in response to *M. tuberculosis* PPD antigen. Positive wells were predefined as containing ≥10 sfu more than, and at least 2 times as many as, negative control wells. The negative control well was required to have <30 sfu.

Statistical Analysis

Odds ratios (ORs) and 95% CIs were calculated for analysis of cross-tabulations. To estimate differences between groups, the χ^2 test was applied with 2-tailed p values. To confirm the results and to use a more accurate test for 2 × 2 tables, we also performed Fisher exact testing. We considered test results with p values <0.05 to be statistically significant.

The recent transmission index (RTI_{n-1}) was calculated as described (*16*). Patients with singleton strains were considered to have TB from reactivation and not recent transmission and, therefore, $RTI_{n-1} = 0$. The average PPD response of patients with singleton strains was considered the baseline. Following calculation of the PPD response of the singleton strains, all recently transmitted strains with a cluster size of 2 were included, and the average PPD response and RTI_{n-1} was re-calculated. This procedure was continued by stepwise inclusion of the next bigger genotypic cluster (i.e., [singleton] + [cluster n = 2] + [cluster n = 3]; [singleton] + [cluster n = 2] + [cluster n = 3] + [cluster n = 4]; ...), and recalculation of the average PPD response for each respective RTI_{n-1} group was performed.

Results

Population Structure of MTBC and Transmission of Isolates

For the study period, 1,003 smear-positive TB cases were identified. Spoligotypes could be obtained from 884 (88%) isolates; many of the strains collected belonged to the *M. africanum* Afri_1 or *M. tuberculosis* sensu stricto lineages, and 86% of all *M. tuberculosis* strains were part of the EA lineage. Therefore, for the remainder of the analysis, we compared all *M. tuberculosis* sensu stricto isolates (including EA), EA lineage isolates separately, and *M. africanum* Afri_1 isolates. For the 2 major lineages (Afri_1 and EA), we identified 17–19 genotypes per 100 isolates, of which 9%–12% were found only once as singletons and thus were most likely the result of reactivation of previous disease (Table 1; Figure 1). The remaining spoligotypes (88%–91%) could be assigned to genotypic clusters with an average size of 11.8 and 13.3 isolates for *M. tuberculosis* EA and *M. africanum* Afri_1, respectively (Table 1). Assuming that recent transmission was correlated with cluster size and that each cluster contained 1 index case, the RTI_{n-1} for both populations indicated that 80%–83% of TB cases were attributable to newly acquired infections.

Despite the similarities between the 2 mycobacterial populations, their individual compositions differed drastically. Whereas 59% of the *M. africanum* population was represented by only a single spoligotype, SIT 181, comprising 198 isolates, the same proportion of the *M. tuberculosis* EA population contained as many as 11–12 smaller clusters of comparable sizes; with SIT 53 being the largest cluster (Figure 1). Consequently, the θ variable, a maximum-likelihood estimate of the genetic diversity of a population (*11*),

Table 1. Spoligotyping results for comparative population structure of *Mycobacterium tuberculosis* sensu stricto (including EA), the EA clade separately, and *M. africanum*, The Gambia, 2002–2009*

	M. tuberculosis sensu stricto		*M. africanum*			
			Afri_1			
Parameters	All lineages	EA clade	With SIT 181	Without SIT 181	Afri_2	Total
Population parameters						
No. isolates (*n*)	548	467	334	136	2	884
No. genotypes	108	79	63	62	2	173
No. singletons (*s*)	60	43	41	41	2	103
No. clusters (*c*)	48	36	22	21	NA	70
Clustering rate, $(n - s)/n$	0.89	0.91	0.88	0.70	NA	0.88
Average cluster size, $(n - s)/c$	10.2	11.8	13.3	4.3	NA	11.2
Genetic diversity	40.0	27.0	22.7	43.4	NA	64.0
Recent transmission index	0.80	0.83	0.81	0.54	NA	0.80

*EA, Euro-American; SIT, shared international type; NA, not applicable.

was lower for *M. africanum* ($\theta = 22.7$) than for *M. tuberculosis* EA ($\theta = 27.0$) or for all *M. tuberculosis* sensu stricto ($\theta = 43.3$). The effect of SIT 181 on the population structure became especially apparent when this cluster was excluded from the analysis, resulting in multiple changes to *M. africanum* population parameters, such as clustering rate, cluster size, and genetic diversity θ (Table 1). The drop in RTI also demonstrates the contribution of SIT 181 to recent transmission within the *M. africanum* Afri_1 lineage.

Spoligoforests of MTBC in West Africa

Using spoligoforests to display mycobacterial populations (*12*) takes the genetic relatedness of spoligotypes into consideration and allows deduction of relationships among bacterial sublineages. When we analyzed the 884 MTBC isolates from The Gambia, we found SIT 53 not only to be the most ancestral *M. tuberculosis* spoligotype but also to constitute the largest *M. tuberculosis* cluster (online Technical Appendix Figure, panel A, wwwnc.cdc.gov/EID/article/19/10/12-1023-Techapp1.pdf). Besides this ancestral strain, we identified 4 more recent major spoligotype clusters (SITs 42, 47, 50, and 61) and 5 third-generation clusters (SITs 20, 60, 62, 144, and 183). Most strains belonged to the modern *M. tuberculosis* lineages. Moreover, when the distribution and size of these individual clusters was considered, *M. tuberculosis* strains seemed to spread evenly within the host population, resulting in a uniformly distributed structure of the bacterial population. In contrast, the *M. africanum* population was highly skewed toward a central cluster of SIT 181, next to which (with the exception of SIT 187) no notable secondary spoligotype clusters emerged. Therefore, the population was concentrated around this spoligotype, and most cases of recently transmitted disease could be attributed to this genotype. Similar results were found when we reanalyzed a published spoligotype dataset from Guinea-Bissau (*6*), the West African country with the highest prevalence of *M. africanum* Afri_1 strains (online Technical Appendix Figure, panel B).

DESTUS Analysis

As indicated by cluster size analysis and RTI_{n-1}, SIT 181 might be responsible for most recently transmitted TB cases in The Gambia. However, inferences about transmission that are purely based on cluster size analysis could be misleading because large clusters could equally be caused by an older strain that has been present for a long time or by strains that mutate slowly (*13*). To account for this imprecision, the DESTUS model was developed to factor the

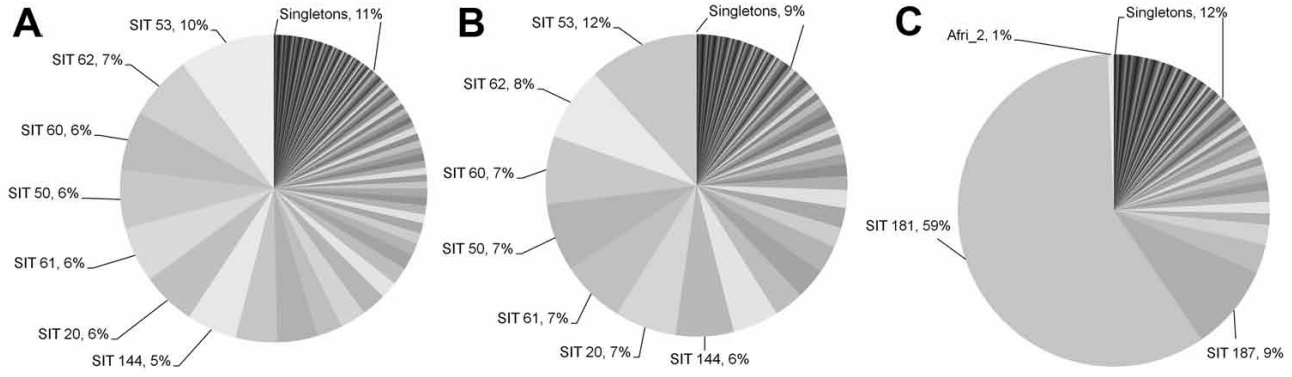

Figure 1. Spoligotyping results showing population structure of *Mycobacterium tuberculosis* and *M. africanum*, The Gambia, 2002–2009. A) All *M. tuberculosis* sensu stricto lineages (including Euro-American); B) Euro-American lineage; C) *M. africanum* lineages (Afri_1 and Afri_2). SIT, shared international type.

mutation rates of spoligotypes into analysis of genotypic clustering as a measure of emerging strains (*13*). DESTUS testing of our dataset for the whole MTBC or stratified by *M. tuberculosis* versus *M. africanum* found that SIT 181 was always detected as a highly significant emerging strain ($p<10^{-30}$–10^{-31}), followed by several other strains (Table 2). Similarly, SIT 181 was the only strain detected as emerging in the dataset from Guinea-Bissau (*6*).

Detecting Temporal Clusters of *M. africanum* SIT 181

To confirm previous results and to detect high rates of temporal clustering, we applied a purely temporal analysis to the *M. africanum* population by using the discrete Poisson model in SaTScan version 9.1.1 (www.satscan.org) (Table 3). We identified a significant (p = 0.001) temporal cluster of SIT 181 cases during August 2007–June 2008. This cluster showed an increased relative risk of 2.65 to the population for contracting SIT 181 when compared with the risk across the full study period (2002–2009).

Immunogenicity of MTBC Isolates

To identify associations between the emergence of certain genotypes and their immunogenicity, we used ELISPOT to measure PPD-induced interferon-γ responses in blood samples collected during 2002–2007 from patients infected with *M. tuberculosis* sensu stricto (n = 235), *M. tuberculosis* EA (n = 194), and *M. africanum* (n = 137). On the basis of the assumption that clustering is indicative of recent transmission, we compared PPD ELISPOT positivity of clustered strains with singletons (Table 4). Our data suggest that *M. africanum* strains clustered by spoligotyping are significantly more likely to produce a positive PPD ELISPOT result than are singletons (OR 31.78, 95% CI 9.24–109.28; p = 0.0001). For *M. tuberculosis* sensu stricto and *M. tuberculosis* EA, we found a similar, yet not significant, tendency.

After applying more stringent criteria than mere clustering, such as determining emerging strains (DESTUS), we compared PPD ELISPOT positivity of SIT 181 to singleton *M. africanum* Afri_1 strains; this analysis confirmed that SIT 181 is significantly more immunogenic than other types (OR 21.09, 95% CI 6.09–73.04; p = 0.0001). Similarly, we found a slight, not significant tendency for patients infected with SIT 181 to be more likely than patients infected with singleton strains to yield a positive Mantoux skin test (OR 1.15, 95% CI 0.30–4.44).

Table 3. Significant temporal clustering of human cases of infection with *Mycobacterium africanum* SIT 181, The Gambia, August 14, 2007–June 3, 2008*

No. cases				
Actual	Expected	Relative risk	LLR	p value
48	21.30	2.65	14.424230	0.001

*Detected with SaTScan version 9.1.1 software (www.satscan.org) (*14*) by applying retrospective purely temporal analysis using the discrete Poisson model. SIT, shared international type; LLR, log-likelihood ratio.

Table 2. Emerging *Mycobacterium tuberculosis* complex strains as detected by DESTUS from samples collected in West Africa*

Study site and years	p value†
Guinea Bissau, 1989–2008	
SIT 181 (Afri_1)	3.8×10^{-8}
SIT 187 (Afri_1)	4.7×10^{-6}
The Gambia, 2002–2009	
SIT 181 (Afri_1)	2.5×10^{-30}
SIT 187 (Afri_1)	5.1×10^{-4}
SIT 60 (*M. tuberculosis*)	1.5×10^{-6}
SIT 61 (*M. tuberculosis*)	2.9×10^{-4}
SIT 183 (*M. tuberculosis*)	3.9×10^{-4}

*Years shown indicate when samples were collected. DESTUS, Detect Emerging Strains of Tuberculosis by Using Spoligotyping (*13*); SIT, shared international type; Afri_1, *M. africanum* West Africa 2.
†After Sidak Dunn correction for type I error.

Discussion

We found a correlation between MTBC strains of higher immunogenicity and their ability to spread within the human host population. In the study population in The Gambia, patients infected with strain SIT 181, the most prevalent *M. africanum* strain, were significantly more likely to yield a positive PPD ELISPOT result than were patients infected with *M. africanum* strains that do not have the ability to establish themselves within the human host population. To describe this association, we constructed a detailed population structure of MTBC isolates in which we analyzed 884 spoligotypes obtained from mycobacterial isolates from TB cases identified during 2002–2009. We found that most circulating strains belonged to either the EA or *M. africanum* Afri_1 clades, and therefore, we specifically focused on the comparison between these 2 lineages. On the basis of the assumption that clustered strains indicate recent transmission, we found that several *M. tuberculosis* strains appeared to have spread evenly within the host population. In contrast, most (59%) *M. africanum* transmission events and infections could be attributed to spoligotype SIT 181, which was responsible for 22% of all TB cases in the country. This result not only confirms recent findings from Guinea-Bissau, in which SIT 181 caused up to 49% of all *M. africanum* infections (*6*), but is also in agreement with a previous study of a smaller set of isolates from this study that showed a similar strain distribution within the 2 major lineages (Afri_1 and EA) (*17*).

Although it is widely accepted that genetic clusters are indicative of recent transmission, caution must be taken with the interpretation of such findings because the successful spread of a strain within the population—and thus genetic clustering—is highly dependent on 2 properties of the bacteria. For successful spread, strains must transmit from infected to uninfected host first; after this initial transmission, the infection must progress to active disease to be transmitted to the next susceptible host. However, only case–contact tracing studies, not molecular clustering data alone, can distinguish between transmission and progression of the

Table 4. PPD ELISPOT results for blood samples from patients infected with *Mycobacterium tuberculosis* sensu stricto (including EA lineage), *M. tuberculosis* EA lineage, and *M. africanum* strains, The Gambia, 2002–2007*

Strain and result	No. spoligotypes			Odds ratio (95% CI)	p value	
	Clustered	Singleton	Total		χ^2 test†	Fisher exact test‡
M. tuberculosis						
Positive	182	19	201	1.65 (0.57–4.77)	0.3495	0.2533
Negative	29	5	34	0.61 (0.21–1.75)	0.3495	0.2533
Total	211	24	235			
M. tuberculosis EA						
Positive	149	14	163	1.58 (0.48–5.15)	0.4479	0.3184
Negative	27	4	31	0.63 (0.19–2.07)	0.4479	0.3184
Total	176	18	194			
M. africanum						
Positive	110	9	119	31.78 (9.24–109.28)	0.0001	0.0001
Negative	5	13	18	0.03 (0.01–0.11)	0.0001	0.0001
Total	115	22	137			
M. africanum	SIT 181					
Positive	73	9	82	21.09 (6.09–73.04)	0.0001	0.0001
Negative	5	13	18	0.05 (0.01–0.16)	0.0001	0.0001
Total	78	22	100			

*PPD, purified protein derivative; EA, Euro-American.
†2-tailed.
‡1-tailed.

different lineages. Consequently, we refer here to the spreading capacity of strains, rather than to transmission.

Our analysis using DESTUS (*13*), which correctly predicted the widely accepted emergence of Beijing lineage *M. tuberculosis* strains in other studies (*13*), detected SIT 181 as an unusually fast-growing strain relative to the mycobacterial background population of the sample. However, DESTUS does not take into account the migration history of Europeans and the mycobacteria they introduced into Africa. Because of this limitation, we sought to confirm our finding with a second approach and conducted a purely temporal analysis. We found that SIT 181 could have been emerging during a certain time in the study period, identifying a temporal cluster during 2007–2008 for which risk for infection with SIT 181 was 2.65-fold higher than that for the whole study period (2002–2009).

With SIT 181 constituting such a prominent cluster, it is conceivable that the strain's high prevalence is related to selective pressure through, for instance, antimicrobial drug therapy. However, because resistance rates are relatively low in The Gambia (*18*), this explanation does not seem to apply. Therefore, we suggest another selective mechanism: we believe differences in spreading capacity and the interaction between *M. tuberculosis* and *M. africanum* populations might play a crucial role. In contrast to *M. africanum*, several clustered strains within the *M. tuberculosis* population have comparable potential to spread within the human host population, but no strain has a notable advantage over another, which results in a well-balanced population structure. We hypothesize that SIT 181, in its expanded ability to spread, resembles these *M. tuberculosis* strains more than it does strains with other spoligotype patterns in the *M. africanum* lineage. Thus, SIT 181 has a selective advantage and is able to compete with *M. tuberculosis* for the same biological niche within the human host.

The nature and extent of epitope variation in *M. tuberculosis* strains is unclear. Findings range from highly variable T-cell epitopes within the *esx* gene family (*19*) to highly conserved epitopes when comparing genetic variation between predicted epitopes and the remainder of the *M. tuberculosis* genome (*7*). The latter study, which described T-cell epitopes as highly conserved, suggests that hyperconservation of T-cell epitopes is beneficial for the bacteria and could result in the successful spread of strains. In our study, we sought to understand whether the magnitude of an induced immune response was correlated with the spreading capacity of the bacteria, with a special focus on SIT 181. We therefore investigated PPD ELISPOT responses of patients infected with either lineage and found a nonsignificant tendency for clustered *M. tuberculosis* sensu stricto and EA strains toward being more likely than singletons to produce a positive PPD result. This small difference in immunogenicity might be in line with our hypothesis that the *M. tuberculosis* population spreads fairly homogenously. Consistent with the large observed differences in spreading capability, SIT 181 or clustered *M. africanum* strains have a 20- to 30-fold higher probability of yielding a positive PPD response ($p<0.0001$). This positive correlation between immunogenicity and spread becomes even more apparent when the RTI is plotted against the average quantitative PPD response (Figure 2). This comparison demonstrates not only the expected larger range in immunogenicity within the *M. africanum* lineage but also the phenotypic relatedness in immunogenicity of the highly spreading strains, independent of lineage.

An association between PPD response and spreading capacity is conceivable. A previous publication found

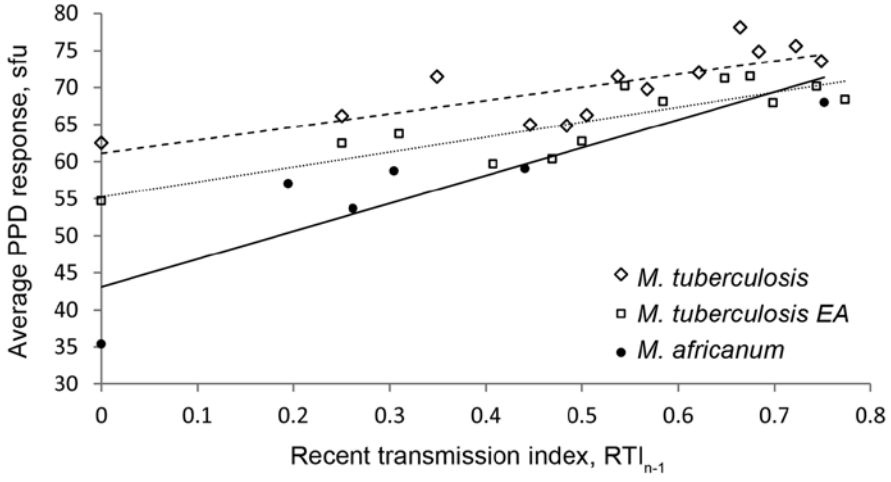

Figure 2. Linear regression analysis showing correlation between average quantitative purified protein derivative (PPD) response and recent transmission index (RTI_{n-1}) for *Mycobacterium tuberculosis* complex isolates, The Gambia, 2002–2007. Open diamonds and dashed line, *M. tuberculosis* sensu stricto, including Euro-American (EA) lineage (R2 = 0.606); open squares and dotted line, *M. tuberculosis* EA lineage (R2 = 0.7272); black circles and solid line, *M. africanum* Afri_1 lineage (R2 = 0.7732). sfu, spot-forming units.

that household contacts who slept in the same bedroom as an index case-patient (i.e., who were exposed to the highest infectious loads) had higher PPD ELISPOT responses than did less-exposed household contacts (*20*). However, further analysis will be needed to conclusively address whether spreading capacity is determined by infectious load; smear-positivity grade of cases; magnitude of the induced immune response; or an as-yet unknown immunogenic protein that enhances transmissibility, the absence of which (from strains of low spread) is merely reflected by a reduced ELISPOT response to PPD.

One possible limitation of spoligotype data is that the technique was designed on the basis of the clustered regularly interspaced short palindromic repeats (CRISPR) regions of *M. tuberculosis* sensu stricto and, therefore, could have a lower resolution when applied to *M. africanum*. Consequently, the large observed SIT 181 cluster could be a result of misclassification. Although the CRISPR regions of *M. tuberculosis* and *M. africanum* have not been extensively compared, we believe misclassification is very unlikely for 2 reasons. First, we found a comparable resolution of the technique for both lineages (18–19 genotypes/100 isolates). Second, when calculating the Hunter-Gaston Index (HGI), a measure for the discriminatory power of a technique, we found that HGI = 0.96 for spoligotyping of *M. tuberculosis*, HGI = 0.94 for *M. africanum* excluding SIT 181, and HGI = 0.64 for *M. africanum* with SIT181. Spoligotyping works equally well for 41% of *M. africanum* isolates and for *M. tuberculosis* isolates (0.94 vs. 0.96) but has drastically worse discriminatory power for the remaining 59% of *M. africanum* strains (0.64). A drop in HGI that was a result of misclassification within the *M. africanum* lineage could only result from a CRISPR region or mutation rate that was notably different between SIT 181 and the other *M. africanum* strains. However, this is unlikely because the remaining strains with HGI = 0.94 evolved out of SIT 181 and, thus, most likely have identical CRISPR regions and mutation rates. Therefore, by comparing these 3 HGI results, we can conclude that SIT 181 is a real cluster and not a result of misclassification. High-resolution genotyping methods, such as mycobacterial interspersed repetitive unit–variable number tandem repeat typing or whole-genome sequencing, is needed to conclusively confirm the genotypic homogeneity of the group of strains that constitute spoligotype pattern SIT 181.

We conclude that spoligotyping possesses comparable discriminatory resolution for *M. tuberculosis* and *M. africanum*. We were able to demonstrate that SIT 181 represents a strain (or family of strains) that clusters genotypically, temporally, and phenotypically and represents a major public health concern in West Africa, responsible for nearly one fourth of TB cases in The Gambia (22%) and Guinea-Bissau (23%) (*6*). Deciphering the virulence mechanisms that determine the differences in immunogenicity and spreading capacity between SIT 181 and the remaining singleton *M. africanum* strains will be key to improving TB prevention and transmission control in this region.

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Plasmodium vivax Malaria during Pregnancy, Bolivia

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Plasmodium vivax is a major cause of illness in areas with low transmission of malaria in Latin America, Asia, and the Horn of Africa. However, pregnancy-associated malaria remains poorly characterized in such areas. Using a hospital-based survey of women giving birth and an antenatal survey, we assessed the prevalence rates of *Plasmodium* spp. infections in pregnant women in Bolivia, and evaluated the consequences of malaria during pregnancy on the health of mothers and newborns. *P. vivax* infection was detected in 7.9% of pregnant women attending antenatal visits, and placental infection occurred in 2.8% of deliveries; these rates did not vary with parity. Forty-two percent of all *P. vivax* malaria episodes were symptomatic. *P. vivax*–infected pregnant women were frequently anemic (6.5%) and delivered babies of reduced birthweight. *P. vivax* infections during pregnancy are clearly associated with serious adverse outcomes and should be considered in prevention strategies of pregnancy-associated malaria.

In Latin America, where malaria transmission is low and mostly unstable, *Plasmodium vivax* is the most prevalent malaria parasite species. Although ≈3 million pregnant women are exposed to malaria in Latin America each year, the actual number of malaria infections during pregnancy is considerably lower (*1*). Pregnancy-associated malaria is poorly characterized in such areas of low or unstable transmission, as in most areas in which of *P. vivax* is predominant (*2*), but malaria can be severe in all parity groups because most women of childbearing age have low levels of prepregnancy and pregnancy-specific protective immunity to malaria (*3*).

One of the first studies that demonstrated parasitization of the placenta by *P. falciparum* was conducted in Latin America (*4*), and reported serious adverse outcomes, such as miscarriages late in pregnancy or stillbirths. No other study related to pregnancy-associated malaria was conducted in Latin America for ≈80 years until a cohort study investigating *P. vivax* infection during pregnancy in Honduras (*5*) and a case-series report of 143 pregnant women infected with *P. falciparum* in French Guiana (*6*) were reported. Both studies outlined serious adverse outcomes (anemia, preterm delivery, hypotrophy, and stillbirth) associated with malaria by parasite species during pregnancy. More recent studies in the Amazon regions of Brazil and Peru reported increased incidence rates of infection with *P. falciparum*, but not *P. vivax*, in pregnant women (*7*,*8*). Outside Latin America, a few studies reported the effect of pregnancy-associated malaria in unstable malaria settings in Africa and Asia (*9*–*12*), and described increased risks for low birthweight and for maternal anemia as consequences of *P. vivax* infection during pregnancy (*13*,*14*).

Using a hospital-based survey of women giving birth and an antenatal survey, we assessed the prevalence rates of *Plasmodium* infection in pregnant women in 2 malaria-endemic areas of Bolivia. We also evaluated the consequences of malaria infection during pregnancy on the health of mothers and newborns.

Patients and Methods

Study Sites

This study was conducted in 2 malaria-endemic areas in Bolivia: the northern district of Guayaramerín in the Amazon region on the border with Brazil, and the district of Bermejo in the southern region on the border with Argentina. In both areas, malaria transmission occurs during the warm and wet season during November–April and is low and unstable; *Anopheles darlingi* and *An. pseudopunctipennis* mosquitoes are the main malaria vectors, respectively (*15*,*16*). *P. vivax* predominates in both areas; *P. falciparum* is present only in Guayaramerín. The annual parasite incidence rates in 2003 were 21.6 and 106.6 infections/1,000 inhabitants in Bermejo and Guayaramerín,

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respectively. These 2 districts are targeted by routine residual insecticide house-spraying programs that use alphacypermethrin (coverage rate <60%). *P. falciparum* isolates are usually resistant to chloroquine and sulfadoxine/pyrimethamine (*17*), but no chloroquine-resistant *P. vivax* has been reported. Ethical approval for this study was obtained from the Bolivian Ministry of Health (National Institute of Health Laboratories, La Paz).

Study Population and Data Collection

Hospital-based Survey

This survey was conducted during December 2002–August 2004 among women giving birth in 2 district hospitals in which >65% of women in the area give birth. Personal history was obtained for all women, including obstetrical antecedents, place of residence, house insecticide spraying, and signed informed consent. After delivery, a placental blood smear was obtained from the maternal side of the placenta. Thick and thin blood films were prepared. Gestational age of neonates was calculated at birth by using the score of Farr et al. (*18*). Newborns were weighted on a digital scale that was accurate to within 10 g.

Antenatal Survey

In Guayaramerín, all consenting pregnant women receiving antenatal care in 2 rural and 5 urban health centers during May 2003–August 2004 were investigated. During each antenatal visit, we performed physical examinations and blood smear examinations for malaria parasites. Giemsa-stained blood smears were read in each center by a trained malariologist. Women with a malaria infection (*P. vivax* or *P. falciparum*) were treated according to the national guidelines at the time (chloroquine or quinine plus clindamycin, respectively). Women were invited to give birth at the district hospital of Guayaramerín and participate in the hospital-based survey.

Laboratory Studies

Hemoglobin levels were determined by using the cyanomethemoglobin method (HemoCue, Cypress, CA, USA). Peripheral and placental smears were stained with Giemsa, and 200 microscopic fields were examined.

Definitions

Neonates were classified as premature if they were <37 weeks gestation at birth. Low birthweight was defined as a body weight <2,500 g. Anemia was defined as a hemoglobin level <11 g/dL, and moderate-to-severe anemia as a hemoglobin level <8 g/dL. Asymptomatic malaria infection was defined as the presence of malaria parasites on blood smears in the absence of fever (axillary temperature >37.5°C) or a history of fever in the preceding 48 hours.

Data Analysis

Twins and stillbirths were excluded from the analysis. *P. vivax* and *P. falciparum* infections were dichotomized independently. Categorical variables were compared by using χ^2 or Fisher exact tests, and continuous variables were compared buy using the Mann-Whitney test. We used Stata/MP 11 (StataCorp LP, College Station, TX, USA) for multiple linear or logistic regressions (with backward stepwise elimination) to adjust for potential confounding variables (mother's age, parity, antenatal care attendance, indoor insecticide spraying, site of study, delivery during transmission season, and sex and gestational age of the baby), and to determine the population attributable fraction (PAF), which is also known as the etiologic fraction, or that proportion of all events (severe anemia, low birthweight) associated with the factor of interest (e.g., *P. vivax* or *P. falciparum* infection).

Results

Hospital-based Survey

During December 2002–August 2004, a total of 1,003 women in Guayaramerín and 504 women in Bermejo had singleton births at the 2 district hospitals. In both hospitals, mean parity and proportion of primiparous women were similar (Table 1). However, women were younger (mean ± SD age 23.2 ± 6.4 years vs. 24.2 ± 6.7 years; p = 0.008) and had more antenatal visits (4.7 ± 2.0 visits vs. 3.7 ± 2.1 visits; p<0.001) in Guayaramerín than in Bermejo. The proportion of women without any antenatal visit was 4 times higher (7.8% vs. 1.9%; p<0.001) in Bermejo than in Guayaramerín. Women lived less often in rural settlements (6.8% vs. 22.7%; p<0.001) and had babies more often during the transmission season (60.0% vs. 52.6%; p = 0.006) in Guayaramerín than in Bermejo. Rates of low-birthweight and moderate-to-severe maternal anemia at birth were similar in both places.

Among 967 women who had babies in Guayaramerín and had a placental smear, 26 (2.7%) were had *P. vivax* infections in placental blood. In addition, 4 (0.4%) had placental *P. falciparum* infections. Among 500 women who had babies in Bermejo and had a placental examination, 15 (3.0%) had *P. vivax* infections in placental blood. Because of these differences, we further adjusted for study area to evaluate the effects of *P. vivax* infection in pregnant women. We further distinguished infections by *P. falciparum* or *P. vivax* for the analysis.

The risk for placental *P. vivax* infection increased during the transmission season in both places (adjusted odds ratio [OR] 2.7, 95% CI 1.3–5.6, p<0.007). There was no effect of parity, mother's age, antenatal care attendance, or indoor insecticide spraying on placental *P. vivax* prevalence in both districts.

Table 1. Baseline characteristics of women and babies at delivery during a hospital-based survey, Guayaramerín and Bermejo Bolivia, 2002–2004*

Characteristic	Guayaramerín, n = 1,003	Bermejo, n = 504	p value
Mothers			
Age, y	23.2 ± 6.4	24.2 ± 6.7	**0.008**
No. previous pregnancies	2.1 ± 2.4	1.9 ± 2.1	0.40
Primiparae	31.7	28.4	0.18
No. antenatal visits	4.7 ± 2.0	3.7 ± 2.1	**<0.001**
No antenatal visit	1.9	7.8	**<0.001**
Houses with indoor insecticide spraying	55.0	50.3	0.098
Women living in rural settlements	6.8	22.7	**<0.001**
Delivery during transmission season	60.0	52.6	**0.006**
Hemoglobin level, g/dL	11.1 ± 2.0	11.4 ± 1.9	**<0.001**
Moderate-to-severe anemia, hemoglobin level <8 g/dL	6.8	6.3	0.70
Babies			
Girls	47.5	49.8	0.41
Birthweight, g	3,310 ± 509	3,383 ± 515	**0.003**
Low birthweight, <2,500 g	5.0	4.8	0.83
Premature babies, <37 weeks	7.6	4.4	**0.018**
Placental *Plasmodium vivax* infection	2.7	3.0	0.73
Placental *P. falciparum* infection	0.4	NA	NA

*Values are mean ± SD or percentage. Significant values (p<0.05) are indicated in **boldface**. NA, not applicable.

Women with placental *P. vivax* infections were more likely than noninfected mothers to have a low-birthweight baby (OR adjusted for study site 3.6, 95% CI 1.4–8.9) (Table 2). These women were also more likely than noninfected women to have moderate-to-severe anemia (adjusted OR 2.5, 95% CI 1.0–6.2).

Factors associated with mean birthweight in a multiple linear regression model are shown in Table 3. Mean birthweight was reduced in premature (–752 g), female (–151 g), first-born (–168 g), and second-born babies (–79 g), as well as in babies born to mothers living in Guayaramerín (–52 g), women who had no antenatal visits (–112 g), and women with placental *P. vivax* infections (–181 g). Mean birthweight was increased in babies born to women >35 years of age (+181 g) or women 25 35 years of age (+102 g). Logistic regression (Table 3) showed that preterm delivery (p<0.001) and placental *P. vivax* infections (OR 6.2, 95% CI 2.2–17.6, p<0.001) were associated with an increased risk for low-birthweight babies. The population attributable risk for low-birthweight babies associated with *P. vivax* infection was 6.1% (95% CI 0.4%–11.4%).

We used a multiple linear regression model to identify factors associated with changes in mean hemoglobin levels (Table 4). Mean hemoglobin level was significantly reduced in multiparous women (–0.28 g/dL; p = 0.012), women in Guayaramerín (–0.38 g/dL; p = 0.001), and women with placental *P. vivax* infections (–0.70 g/dL; p = 0.026). Logistic regression showed that placental *P. vivax* infection remained independently associated with an increased risk for moderate-to-severe anemia (OR 2.5, 95% CI 1.04–6.2, p = 0.04). The population attributable risk for moderate-to-severe maternal anemia associated with *P. vivax* infection was 3.5% (95% CI –1.2% to 8.1%).

In contrast to placental *P. vivax* infection, placental *P. falciparum* infection was more likely to occur in primiparous women than in multiparous women (0.7% vs. 0.1%; p = 0.05). After exclusion of Bermejo and *P. vivax* infections, the risk for low birthweight increased in premature babies (OR 31.2, 95% CI 15.7–62.1; p<0.001) and in babies born to mothers with placental *P. falciparum* infections (OR 5.1, 95% CI 1.6–16.6; p = 0.006).

Antenatal Survey

During May 2003–August 2004, a total of 359 women had antenatal visits and subsequently gave birth in Guayaramerín. Mean ± SD parity was 1.9 ± 2.2 (range 0–13), mean ± SD age was 22.8 ± 6.2 years (range 13–45 years), mean ± SD number of antenatal visits was 4.9 ± 1.8 (range 1–10), and mean ± SD number of blood screenings was 3.4 ± 1.9 (range 1–9). Of these women, 330 had no documented malaria episodes, 1 was infected with *P. falciparum*, and 28 (7.8%; 95% CI 5.0–10.6) had ≥1 *P. vivax*

Table 2. Risks for low birthweight and maternal anemia associated with placental *Plasmodium vivax* infections, Guayaramerín and Bermejo, Bolivia, 2002–2004*

Risk	OR for adverse condition (95% CI), p value		Adjusted OR† (95% CI), p value
	Bermejo	Guayaramerín	
Low-birthweight babies of women with or without placental infection	3.4 (0.7–16.1), 0.10	3.7 (1.2–11.2), **0.01**	**3.6 (1.4–8.9), 0.003**
Moderate-to-severe anemia among women with or without placental infection	4.3 (1.1–16.4), **0.02**	1.8 (0.5–6.1) 0.35	**2.5 (1.0–6.2), 0.03**

*OR, odds ratio. Significant values (p<0.05) are indicated in **boldface**.
†Adjusted OR after stratifying for study sites.

Table 3. Factors associated with mean birthweight and risk for low birthweight babies, excluding *Plasmodium falciparum* infections, during hospital-based survey, Guayaramerín and Bermejo, Bolivia, 2002–2004*

	Multiple linear regression, n = 1,417		Multivariate logistic regression, n = 1,417	
Characteristic	Adjusted difference in mean birthweight, g (95% CI)†	p value	Adjusted OR for low birthweight (95% CI)	p value
Baby				
Mature	0	NS	1	NS
Premature	–752 (–849 to –656)	**<0.001**	37.8 (20.9–68.3)	**<0.001**
Boy	0	NS	1	NS
Girl	–151 (–198 to –105)	**<0.001**	1.7 (0.98–3.1)	0.06
Mother				
Multiparous	0	NS	1	NS
Secondiparous	–79 (–146 to –13)	**0.02**	1.6 (0.91–2.9)‡	0.10
Primiparous	–168 (–232 to –103)	**<0.001**	NS	NS
<25 y of age	0	NS	NS	0.56
25–35 y of age	102 (38–167)	**0.002**	NS	NS
>35 y of age	181 (83–279)	**<0.001**	NS	NS
Antenatal visit	0	NS	NS	0.22
No antenatal visit	–112 (–235 to 10)	0.07	NS	NS
Bermejo	0	NS	NS	0.47
Guayaramerín	–52 (–102 to –2)	**0.04**	NS	NS
Noninfected placenta	0	NS	1	NS
P. vivax–infected placenta	–181 (–321 to –41)	**0.01**	6.2 (2.2–17.6)	**<0.001**

*Significant values (<0.05) are indicated in **boldface**. Multivariate models adjusting for mother's age, number of previous pregnancies, antenatal visits, houses with indoor insecticide spraying, site of study, delivery during transmission season, mother's anemia, and sex and gestational age of the baby. Only significant variables (p<0.10) from linear regression model are shown. The same variables were used for the logistic regression model. OR, odds ratio; NS, not significant.
†Baseline mean birthweight was 3,537 g.
‡First and second pregnancies combined compared with multiparous women.

infection between first antenatal visit and delivery (20 women had 1 infection, 6 had 2 infections, and 2 had 3 infections). Of these 28 women, 57.5% had febrile illness and 42.5% were asymptomatic. A total of 143 women (42.8%; 95% CI 37.5%–48.1%) had anemia and 23 (6.9%; 95% CI 4.1–9.6) had moderate-to-severe anemia. Fourteen women (3.9%, 95% CI 1.9%–5.9%) gave birth to low-birthweight babies. The proportion of women with *P. vivax* infections was similar in primiparae (7.3%) and multiparae (8.1%). The *P. vivax* infection rate was 4.3% (3/69), 4.6% (10/215), and 6.5% (22/336) during the first, second, and third trimesters, respectively.

After logistic regression, the risk for moderate-to-severe anemia at delivery remained associated with parity and was higher in multiparae than in primiparae (OR 3.9, 95% CI 1.1–13.6; p = 0.03) and in women with *P. vivax* infection during antenatal visits (OR 3.7, 95% CI 1.2–11.1; p = 0.02). The proportion of low-birthweight babies was higher in women who had been infected with *P. vivax* during pregnancy (17.9%) than in noninfected women (2.7%; p<0.001). The odds of low-birthweight babies born to mothers without *P. vivax* infection, with 1 infection, and with ≥2 infections during the antenatal survey were 2.8%, 17.6%, and 33.3%, respectively (p<0.001, by score test for trend of odds). The mean birthweight of babies born to women who had been infected with *P. vivax* during pregnancy was 289 g lower than that of babies born to noninfected mothers (mean ± SD 3,054 ± 535 g vs. 3,343 ± 480 g; p = 0.008). The mean hemoglobin level for women who were infected with *P. vivax* during pregnancy was 0.74 g/dL lower than that for noninfected mothers (mean ± SD 10.3 ± 1.9 g/dL vs. 11.0 ± 2.1 g; p = 0.06).

Factors associated with a change in mean birthweight by a multiple linear regression model are shown

Table 4. Factors associated with mean hemoglobin level and risk for moderate-to-severe anemia, excluding *Plasmodium falciparum* infections, during hospital-based survey, Guayaramerín and Bermejo, Bolivia, 2002–2004*

	Multiple linear regression, n = 1,439		Logistic regression, n = 1,439	
Characteristic	Adjusted difference in mean hemoglobin level, g/dL (95% CI)†	p value	Adjusted OR for moderate-to-severe anemia (95% CI)	p value
Primiparous mother	0	NS	NS	0.23
Multiparous mother	–0.28 (–0.51 to –0.06)	**0.012**	NS	NS
Bermejo	0	NS	NS	0.42
Guayaramerín	–0.38 (–0.59 to –0.16)	**0.001**	NS	NS
Noninfected placenta	0	NS	1	NS
P. vivax–infected placenta	–0.70 (–1.32 to –0.09)	**0.026**	2.5 (1.04–6.2)	0.04

*Significant values (<0.05) are indicated in **boldface**. Multivariate models adjusting for number of previous pregnancies, antenatal visits, houses with indoor insecticide spraying, site of study, and delivery during transmission season. Only significant variables (p<0.10) from the linear regression model are shown. The same variables were used for the logistic regression model. OR, odds ratio; NS, not significant.
†Baseline mean hemoglobin level was 11.7 g/dL.

in Table 5. Mean birthweight was lower in girls (–135 g), premature babies (–426 g), and first-pregnancy babies (–181 g), as well as in babies born to anemic mothers (–92 g) or to mothers infected with *P. vivax* during pregnancy (–266 g). Logistic regression showed that preterm delivery (p = 0.001) and *P. vivax* infection during pregnancy (OR 8.8, 95% CI 2.4–32.5) were associated with low birthweight.

Discussion

Long after the first studies on pregnancy-associated malaria conducted in Africa, most studies in Latin America during the past decade, mainly case-series studies, reported numerous adverse conditions, such as a high frequency of maternal anemia, miscarriage, stillbirth, preterm delivery, and low birthweight (*6*,*19*–*23*), related to malaria infections with *P. falciparum* or *P. vivax* during pregnancy. As observed in other malaria-endemic areas, a cohort study in Peru and a cross-sectional study in Brazil reported a 2.5-fold increase in susceptibility to *P. falciparum* malaria among pregnant women than among nonpregnant women (*7*,*8*). Neither study demonstrated a similar higher frequency of *P. vivax* infection in pregnant women.

In the current study, *P. vivax* infection was detected in 7.9% of pregnant women attending antenatal visits. This proportion is similar to rates in other settings, such as in Thailand (6.4%–8.5%) and Honduras (9.1%) (*5*,*9*,*13*). These findings suggest a constant proportion of *P. vivax* infections during pregnancy in different malaria transmission patterns. In Thailand, 23% of all *P. vivax* malaria episodes were symptomatic (*13*), but this rate reached 42% in Bolivia. In addition to possible differences in background immunity resulting from more unstable transmission in Bolivia, this difference might also be caused by prompt diagnosis and treatment on a weekly basis in the study in Thailand, which enabled parasite detection and cure before onset of symptoms. In our study, diagnosis and treatment were performed monthly, which is the approximate interval between 2 antenatal visits, which enabled a longer time for symptoms to develop.

Among pregnant women, primiparae women are most vulnerable to *P. falciparum* infections, and the difference between primiparae and multiparae women is more pronounced in areas of stable than unstable malaria transmission (*11*,*12*,*24*). We observed similar differences, despite a limited number of *P. falciparum*–infected women. In contrast and consistent with previous reports (*10*), *P. vivax* infection was observed in a similar proportion of women of all parities. However, 1 study reported an increased risk for *P. vivax* infection in primiparae than in multiparae (*13*).

A high proportion of pregnant women in both study sites in Bolivia had anemia, and the proportion of women with moderate-to-severe anemia increased with parity. As observed in unstable malaria transmission settings, the risk for maternal anemia was more pronounced in multiparae than in primiparae women (*9*,*10*,*12*). In our study, *P. vivax* infection was associated with a reduction of 0.7 g/dL in the hemoglobin level of infected pregnant women than that of noninfected women. A similar difference (0.8 g/dL) was observed in Honduras (*5*) between *P. vivax*–infected and noninfected women. Logistic regression showed that the risk for maternal anemia was associated with *P. vivax* infection at delivery, multiparity, and the study district in northern Bolivia. In our antenatal cohort study, *P. vivax* infection acquired during pregnancy remained independently associated with the risk for moderate-to-severe anemia. A similar relationship was observed in Thailand (*13*). Other studies also reported the effect of infection with *P. falciparum* or *P. vivax* during pregnancy on the risk for maternal anemia, but confounding malaria species could have led to classification bias (*9*,*10*,*12*,*19*).

Table 5. Factors associated with mean birthweight and risk for low birthweight during antenatal survey, Guayaramerín, Bolivia, 2003–2004*

	Multiple linear regression, n = 329		Logistic regression, n = 329	
Characteristic	Adjusted difference in mean birthweight, g (95% CI)†	p value	Adjusted OR for low birthweight (95% CI)	p value
Baby				
Mature	0	**<0.001**	1	NS
Premature	–426 (–626 to –227)	NS	10.5 (2.8–39.8)	**0.001**
Boy	0	NS	NS	0.98
Girl	–135 (–237 to –34)	**0.009**	NS	0.98
Multiparous mother	0	NS	NS	0.52
Primiparous mother	–181 (–287 to –75)	**0.001**	NS	0.52
No anemia at delivery	0	**0.08**	NS	0.69
Anemia at delivery	–92 (–195 to 10)	0.08	NS	0.69
Not infected at antenatal visits	0	NS	1	NS
Infected with *Plasmodium vivax* at antenatal visits	–266 (–453 to –78)	**0.006**	8.8 (2.4–32.5)	**0.001**

*Significant values (<0.05) are indicated in **boldface**. Multivariate models adjusting for mother's age, maternal anemia, number of previous pregnancies, and sex and gestational age of the baby. Only significant variables (p<0.10) from the linear regression model are shown. The same variables were used for logistic regression model. OR, odds ratio; NS, not significant.
†Baseline mean birthweight was 3,538 g.

Babies born to *P. vivax*–infected mothers showed a major mean birthweight reduction of 181 g when compared with babies born to noninfected women, which is consistent with observations in Thailand and Honduras (107 and 198 g, respectively) (*5*,*13*). Mean birthweight was also highly reduced in case of preterm delivery, of poor antenatal clinic attendance, and in babies born to first- and second-pregnancy. These factors were consistently identified in studies performed in unstable malaria settings (*10*,*13*). In our study, placental *P. vivax* infection was associated with a 6-fold higher risk for low birthweight, which is ≈4 times higher than the risk estimated in Thailand (*13*). However, in Madagascar, in areas of unstable malaria transmission, the risk for low birthweight associated with *P. falciparum* infection was 2.5 times that in areas with stable transmission (*11*). As suggested by the higher proportion of symptomatic infections in our study in Bolivia, the index of stability may be lower in Bolivia than in Asia if one takes into account a higher risk for low birthweight associated with *P. vivax* infection. In our study, the risk for low birthweight increased with the number of *P. vivax* infections that occurred during pregnancy (by test for trend). These data are consistent with a similar dose-dependent effect in a study in Thailand, which reported a greater reduction in birthweight in mothers infected ≥5 times than in mothers infected only 1 time (*9*).

In Bolivia, the PAF of moderate-to-severe anemia associated with *P. vivax* malaria was 3.5%, and the PAF for low birthweight was 6.1% for *P. vivax*. Our estimation is consistent with the 2%–15% estimation of the PAF for severe anemia related to *P. falciparum* in malaria-endemic areas (*25*). In contrast, *P. vivax* seemed to have less of an effect on the risk for low birthweight than *P. falciparum* in malaria-endemic areas in Africa (risk 8%–14% estimated by Steketee et al.) (*25*).

Our cross-sectional survey has limitations, including selection biases (if most women do not attend selected structures or because they give birth at home because private and nongovernment organization sectors predominate in the public sector) and representativeness. In the 2 districts we studied, private and nongovernment organization sectors are negligible and most births are in public sector facilities. However, ≈25%–33% of births were at home in the regions studied. This factor is a possible limitation because we did not assess deliveries at home. This limitation is similar for prenatal visits, but the number of pregnant women who receive prenatal care in Bolivia is high (>80%). We conducted the prenatal follow-up study in 7 health centers to ensure representativeness. To avoid missing the transmission season, we conducted the study in >1 calendar year.

Although *P. vivax* infections are clearly associated with serious adverse outcomes during pregnancy, accumulation of *P. vivax* in the placenta has not been reported. *P. vivax*–infected erythrocytes can bind chondroitin sulfate A, the placental binding receptor (*26*), but at a 10-fold lower level than *P. falciparum*–infected erythrocytes (*27*), and at a similar level in isolates from pregnant women or nonpregnant persons (*28*). The paucity of *P. vivax* in the placenta has been reported (*29*,*30*), and *P. vivax* has been inconsistently associated with the presence of malaria pigment in the placenta, but not associated with placental pathologic changes (*14*,*31*). High circulating levels of inflammatory cytokines during the paroxysms of *P. vivax* malaria (*32*) may be sufficient to impair fetal growth and cause maternal anemia, as hypothesized by Nosten et al. (*2*). Moreover, rosette formation is a frequent cytoadhesive phenotype in *P. vivax* infections and has been associated with an increased risk for anemia (*28*). Nevertheless, phenomena involved in pathologic mechanisms specific for *P. vivax* infection during pregnancy remain to be elucidated.

P. falciparum and *P. vivax* infections during early pregnancy have been shown to result in impaired fetal growth (*33*), which emphasizes the need to include early pregnancy in the prevention strategies of pregnancy-associated malaria. In addition, almost half of *P. vivax* infections were asymptomatic, suggesting that women should be screened for malaria at every antenatal clinic visit, and treated if test results were positive. Although the effects of *P. vivax* infection during pregnancy have become increasingly documented, health personnel in malaria-endemic areas of Latin America still largely ignore recommendations for diagnosis and treatment of malaria in pregnant women (*34*). Efforts should be undertaken to increase staff training to limit the effect of malaria during pregnancy.

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Antibody Responses against *Pneumocystis jirovecii* in Health Care Workers Over Time

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In a previous cross-sectional study, we showed that clinical staff working in a hospital had significantly higher antibody levels than nonclinical staff to *Pneumocystis jirovecii*. We conducted a longitudinal study, described here, to determine whether occupation and self-reported exposure to a patient with *P. jirovecii* pneumonia were associated with antibody levels to *P. jirovecii* over time. Baseline and quarterly serum specimens were collected and analyzed by using an ELISA that targeted different variants of the *Pneumocystis* major surface glycoprotein (MsgA, MsgB, MsgC1, MsgC3, MsgC8, and MsgC9). Clinical staff had significantly higher estimated geometric mean antibody levels against MsgC1 and MsgC8 than did nonclinical staff over time. Significant differences were observed when we compared the change in antibody levels to the different MsgC variants for staff who were and were not exposed to *P. jirovecii* pneumonia–infected patients. MsgC variants may serve as indicators of exposure to *P. jirovecii* in immunocompetent persons.

Pneumocystis jirovecii pneumonia (PCP) is the leading AIDS-defining illness in the United States and is a serious complication in transplant recipients and other immunocompromised persons. Although understanding of the epidemiology and transmission of *Pneumocystis* spp. has increased, much remains unknown. Studies have demonstrated the ubiquity of *Pneumocystis* isolates in the environment and their presence in the human lung; however, little is known about the precise reservoir for the *Pneumocystis* species that infects humans (*P. jirovecii*) (*1*–*4*). Traditionally, PCP was thought to occur by reactivation of latent infection acquired during childhood, but more recent studies suggest that the disease can also occur after recent exposure and infection (*5*–*12*). Animal-to-animal airborne transmission of *Pneumocystis* organisms has been demonstrated after brief periods of exposure (*13*–*16*). These animal studies suggest that person-to-person airborne transmission can take place after brief periods of exposure.

The occurrence of PCP outbreaks in hospital and clinic settings supports the theory that *P. jirovecii* can be transmitted from a patient with PCP to an immunocompromised patient at risk for PCP (*17*,*18*). Studies have also demonstrated that immunocompetent hospital workers who care for patients with PCP can become colonized with *P. jirovecii* as can family members of PCP-infected patients (*19*,*20*). Animal studies have shown that immunocompetent mice become transiently colonized with *Pneumocystis* spp. after exposure to immunocompromised PCP-infected mice and that the colonized mice subsequently transmit and infect *Pneumocystis*-free immunocompromised mice (*21*). These findings suggest that colonized persons who do not have PCP may be another source of transmission.

In the absence of a culture method, seroepidemiologic studies have provided valuable insights into the epidemiology of *P. jirovecii* in humans. In our prior studies, we used an ELISA to measure IgG levels against the *P. jirovecii* major surface glycoprotein (Msg) (*22*,*23*). The ELISA identifies overlapping recombinant fragments that span the length of the Msg from the amino terminus (MsgA) to the middle portion (MsgB) to the carboxyl terminus (MsgC) (*22*). In an earlier cross-sectional study of hospital staff with clinical (with patient contact) and nonclinical (without patient contact) occupations, staff with clinical occupations had significantly higher serum antibody levels against the MsgC1 variant, but not against MsgA or MsgB, than

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did staff with nonclinical occupations (*23*). To examine antibody responses in these same health care workers over time, we conducted a longitudinal study of clinical and nonclinical hospital staff in which serial serum specimens were collected. These specimens were then analyzed for IgG against MsgA, MsgB, and MsgC1 and against 3 additional Msg constructs: MsgC3, MsgC8, and MsgC9. The study objectives were to determine whether clinical occupation and self-reported exposure to a patient with PCP were associated with higher antibody levels against the different Msg variants over time. Such findings would support the theory that patient-to-provider transmission of *Pneumocystis* isolates occurs in the hospital setting and address the use of antibody levels against Msg as epidemiologic markers of *Pneumocystis* infection.

Methods

Participants

A convenience sample of 115 San Francisco General Hospital (San Francisco, CA, USA) health care workers was enrolled in the longitudinal study from January 2007 through February 2009. HIV/AIDS Division and Division of Pulmonary and Critical Care Medicine staff were sought preferentially because they worked most consistently with patients who were infected with HIV and/or PCP, the presumed reservoirs of *P. jirovecii*. Recruitment was conducted primarily by word of mouth; emails were sent to departmental listservs; and announcements were made at staff meetings, medical conferences, and orientations for medical students and residents. At study entry, no respondents reported PCP infection. Participants who provided serial (2–5) serum samples were included in this analysis; these persons are a subset of the participants included in our prior cross-sectional study (*23*). The University of California, San Francisco, and the University of Cincinnati Institutional Review Boards approved this study. All participants provided written, informed consent.

Questionnaires

Participants completed an initial baseline questionnaire at enrollment and a follow-up questionnaire every 4–8 weeks. Questions about demographic characteristics and medical history (i.e., cigarette smoking, chronic lung disease, and immunocompromising condition) were asked on the initial questionnaire. The questions regarding chronic lung disease and immunocompromising condition were purposefully generalized, and specification of any condition was made optional to protect confidentiality regarding a participant's medical history. On both the initial and follow-up questionnaires, participants were asked whether they had ever been exposed to a patient with PCP and, if so, when their most recent exposure occurred. Participants were asked to choose from the following possible answers: within 1 hour, within 24 hours, within 7 days, within 1 month, and >1 month ago.

Classification of Participants

Participants were placed in the following 2 categories: health care workers with patient contact and health care workers without patient contact, as described (*23*). Health care workers with patient contact (clinical staff) were persons who worked directly with patients in a clinical or research setting. Clinical staff included direct care providers, such as attending physicians, fellows, interns/residents, medical students, nurse practitioners, and nurses, as well as ancillary clinic staff, such as medical assistants, social workers, pharmacists, and clinical research assistants. Health care workers without patient contact (nonclinical staff) were persons who worked at the hospital but who did not work directly with patients in either a clinical or a research setting. Nonclinical staff included administrative and laboratory staff.

Serum ELISA

Serum specimens were collected at the time of enrollment and quarterly (equal to a median of 3.2 months after the previous visit [interquartile range 3.0–3.5 months]) for up to 1 year, or for as long as each health care worker participated in the study. From each participant, 2–5 serum specimens were collected. All serum specimens were stored at -80°C and subsequently sent to the University of Cincinnati (Cincinnati, OH, USA) for analysis. An ELISA that included recombinant fragments derived from a single *P. jirovecii* Msg isoform was used to measure IgG levels (*22*). IgG responses against MsgA, MsgB, MsgC1, MsgC3, MsgC8, and MsgC9 were measured. All serum specimens from the same participant and a standard reference serum specimen were placed in duplicate wells in a 96-well plate and were tested against all 6 Msg fragments. Phosphate-buffered saline without antigen was used as a negative control. Reactivity was corrected by subtracting the reactivity of the serum to the phosphate-buffered saline from the reactivity of the serum to the antigen, and results were quantified by the method of Bishop and Kovacs (*24*). A standard reference serum specimen with specificity for each Msg construct was prepared by mixing serum from 4–6 specimens with high reactivity for the specific construct. These specimens were selected from testing banks of serum specimens from HIV-infected patients and healthy blood donors. From this, a standard curve was generated for each Msg construct on each day the assay was used. This curve was used to calculate the units of reactivity to the Msg construct. For each standard serum pool, we assigned a value of 100 units of reactivity to its target Msg construct in 100 μL of a 1:100 dilution. Test

serum samples were diluted at 1:100–1:200 to fit the linear portion of the standard curves. Taking into account the dilution, we then calculated units of reactivity. The lowest possible value of 1 U was assigned to all specimens with values below the standard curve.

Statistics

Demographic, health, and professional characteristics were compared by occupation (clinical vs. nonclinical). Simple categorical variables (sex, ethnicity, health conditions, exposure to PCP-infected patients) were compared by using the χ^2 test. Fisher exact test was used when expected counts were <5. Multicategorical variables (race, work department) were compared by using logistic regression. Continuous variables (age) were compared by using the Student *t* test. Antibody levels were normalized by using a log transformation; results were exponentiated and presented as estimated geometric means (EGMs) with 95% CIs. Tobit mixed model regression for censored data was used to estimate the difference between antibody response in clinical staff and that in nonclinical staff. For a subset of workers who self-identified as having been exposed to a PCP-infected patient within 1 month before or after having a study serum specimen drawn, the changes in antibody levels from the time of exposure to 3 months and 6 months afterward were calculated and compared with changes from baseline to subsequent serum antibody levels in workers with no known *P. jirovecii* exposure. We compared antibody changes within each group using paired *t* tests and compared differences between the groups using a general linear model with 3-month or 6-month change as the dependent variable. Statistical significance was defined as $p<0.05$. All calculations were performed with SAS software 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

Participants

We enrolled 115 staff members, and each staff member provided at least 2 serum specimens. Participants ranged from 22 to 80 years of age (mean 39.5 years), and 66 (57.4%) were female (Table 1). Seventy (60.9%) participants were White/Caucasian, 30 (26.1%) were Asian, and 3 (2.6%) were Black/African American. Seventeen (14.8%) were ethnically Hispanic/Latino. Thirty-nine (33.9%) participants had smoked at least 100 cigarettes in their lifetime; 19 (16.5%) had an underlying lung condition; and 8 (7.0%) had an immunocompromising condition. Fifty-two (45.2%) participants were part of the HIV/AIDS Division, 30 (26.1%) were part of the Division of Pulmonary and Critical Care Medicine (CCM), 27 (23.5%) were part of the Department of Medicine, and 6 (5.2%) were members of other departments (Obstetrics and Gynecology, Psychiatry, and Radiology). Of the 115 participants, 79 (68.7%) had a known exposure to a PCP-infected patient before the study period.

Participant Classification by Occupation

Ninety-nine (86.1%) participants were categorized as clinical staff, and 16 (13.9%) were categorized as nonclinical staff (Table 1). No significant differences were found between clinical and nonclinical staff in age, sex, race, ethnicity, smoking habits, underlying lung condition, or

Table 1. Characteristics of San Francisco General Hospital staff in a study of antibody responses to *Pneumocystis jirovecii*, San Francisco, California, USA, 2007–2009*

Characteristic	Total no. (%), N = 115	Clinical, no. (%), n = 99	Nonclinical, no. (%), n = 16	p value
Demographic				
Mean age ± SD, y	39.5 ± 12.1	39.0 ± 12.4	42.8 ± 9.7	0.25
Sex				
F	66 (57.4)	56 (56.6)	10 (62.5)	Ref.
M	49 (42.6)	43 (43.4)	6 (37.5)	0.66
Race				
White/Caucasian	70 (60.9)	59 (59.6)	11 (68.8)	Ref.
Asian	30 (26.1)	28 (28.3)	2 (12.5)	0.14
Black/African American	3 (2.6)	2 (2.0)	1 (6.3)	0.32
Multiple/other	12 (10.4)	10 (10.1)	2 (12.5)	0.95
Ethnicity				
Hispanic/Latino	17 (14.8)	13 (13.1)	4 (25.0)	0.25
Health condition				
Ever smoked	39 (33.9)	31 (31.3)	8 (50.0)	0.14
Lung condition	19 (16.5)	14 (14.1)	5 (31.3)	0.14
Immune condition	8 (7.0)	4 (4.0)	4 (25.0)	0.01
Department/Division				
HIV/AIDS	52 (45.2)	44 (44.4)	8 (50.0)	Ref.
Pulmonary and Critical Care Medicine	30 (26.1)	27 (27.3)	3 (18.8)	0.49
Medicine	27 (23.5)	23 (23.2)	4 (25.0)	0.90
Other	6 (5.2)	5 (5.1)	1 (6.3)	0.81
Ever exposed to PCP patient	79 (68.7)	77 (77.8)	2 (12.5)	<0.001

*Ref., reference category; PCP, *Pneumocystis jirovecii* pneumonia.

department/division. However, a significantly greater proportion of nonclinical staff than clinical staff reported having an immunocompromising condition (25.0% vs. 4.0%; p = 0.01). As expected, a significantly greater proportion of clinical staff than nonclinical staff reported exposure to a PCP-infected patient (77.8% vs. 12.5%; p<0.001).

EGM Antibody Levels against Msg Over Time by Occupation

All staff had detectable antibodies against all Msg fragments: MsgA, MsgB, MsgC1, MsgC3, MsgC8, and MsgC9 (Table 2). For this study, the 99 clinical staff members provided a total of 396 serum specimens (mean 4 specimens), and the 16 nonclinical staff members provided 80 serum specimens (each provided 5 specimens). Overall, when levels of clinical and nonclinical staff were compared, clinical staff had significantly higher EGM antibody levels against MsgC1 (EGM 38.4 vs. 19.8; adjusted p = 0.004) and MsgC8 (EGM 46.0 vs. 27.6; adjusted p = 0.02), even when we controlled for age and immunocompromising condition. EGM antibody levels to MsgA, MsgB, MsgC3, and MsgC9 were not significantly different between clinical and nonclinical staff.

Change in EGM Antibody Levels against Msg Over Time in PCP-exposed and Never PCP-exposed Staff

To evaluate the relationship between PCP exposure and antibody response over time, we identified participants who reported being exposed to a PCP-infected patient within 1 month before any serum collection, irrespective of their occupation (exposed, n = 37), and compared their antibody levels at the time of exposure with their antibody levels 3 and 6 months later. As a control comparison, baseline serum antibody levels of never PCP-exposed persons (never exposed, n = 20), were compared with their antibody levels 3 and 6 months later. Thirty-three of the 37 (89%) exposed participants had been exposed to PCP-infected patients repeatedly over the study period. Members of the PCP-exposed group showed no significant difference in EGM antibody levels after PCP exposure for any Msg variant over time (Figure 1, panels A–F). In contrast to the findings in the PCP-exposed group, EGM antibody levels against MsgC1 in the never PCP-exposed group declined significantly after 3 months (mean change –2.87, 95% CI –5.74 to –0.01; p = 0.049) (Figure 1, panel C). Declines in EGM antibody levels against MsgC3 and MsgC8 over 3 months were of borderline significance (mean change –7.26, 95% CI –14.7 to 0.18; p = 0.06; and mean change –4.30, 95% CI –8.73 to 0.13; p = 0.06; respectively) (Figure 1, panels D, E). However, no significant differences were found in MsgA (Figure 1, panel A), MsgB (Figure 1, panel B), or MsgC9 (Figure 1, panel F) during this time.

Mean changes in EGM antibody levels within the PCP-exposed group were then compared with mean changes in the never PCP-exposed group (Figure 2, panels A–F). No difference was found in EGM antibody levels at baseline between exposed (antibodies measured at the time of exposure) and never-exposed (antibodies measured at the time of baseline enrollment) participants. In contrast, the difference in mean change was significant after 3 months for MsgC1 (mean change 1.67 vs. –2.87; p = 0.04) (Figure 2, panel C), after 3 and 6 months for MsgC3 (mean change 4.09 vs. –7.26, p = 0.02 and 5.10 vs. –8.24, p = 0.03, respectively) (Figure 2, panel D), after 3 and 6 months for MsgC8 (mean change 2.29 vs. –4.30, p = 0.02 and 1.71 vs. –3.30, p = 0.048, respectively) (Figure 2, panel E), and after 6 months for MsgC9 (mean change 1.67 vs. –3.11, p = 0.03) (Figure 2, panel F). After we adjusted for age and an immunocompromising condition, mean change after 6 months in MsgC1 became significant (mean change 1.31 vs. –3.43, p = 0.02). However, mean changes in MsgC3 and MsgC8 lost statistical significance. In contrast, no significant differences were found between the exposed and never-exposed groups in mean change for MsgA (mean change after 3 months 1.50 vs.1.46, p = 0.09; mean change after 6 months 0.04 vs.2.51, p = 0.30, respectively) (Figure 2, panel A), MsgB (mean change after 3 months –0.10 vs. –0.10, p = 1.00; mean change after 6 months 0.02 vs. 0.10, p = 0.93; respectively) (Figure 2, panel B), or MsgC9 after 3 months (mean change 1.14 vs. –0.35, p = 0.43) (Figure 2, panel F).

Discussion

This study demonstrates that health care worker occupation is associated with antibody levels against *P. jirovecii* MsgC variants overall and that self-reported exposure

Table 2. Antibody levels against Msg, San Francisco General Hospital staff, San Francisco, California, USA, 2007–2009*†

Antibody	Occupation, estimated geometric mean (95% CI)		p value
	Clinical, n = 396	Nonclinical, n = 80	
MsgA	12.0 (11.1–12.9)	18.4 (17.1–19.9)	0.45
MsgB	4.9 (4.6–5.1)	6.7 (6.3–7.1)	0.56
MsgC1	38.4 (35.9–41.1)	19.8 (18.6–21.2)	0.004
MsgC3	77.1 (71.9–82.7)	52.9 (49.4–56.8)	0.09
MsgC8	46.0 (42.9–49.2)	27.6 (25.8–29.6)	0.02
MsgC9	34.6 (32.7–36.6)	27.0 (25.5–28.5)	0.17

*Msg, major surface glycoprotein.
†By occupation for all time points and for all participants, adjusted for age and immune disorder,

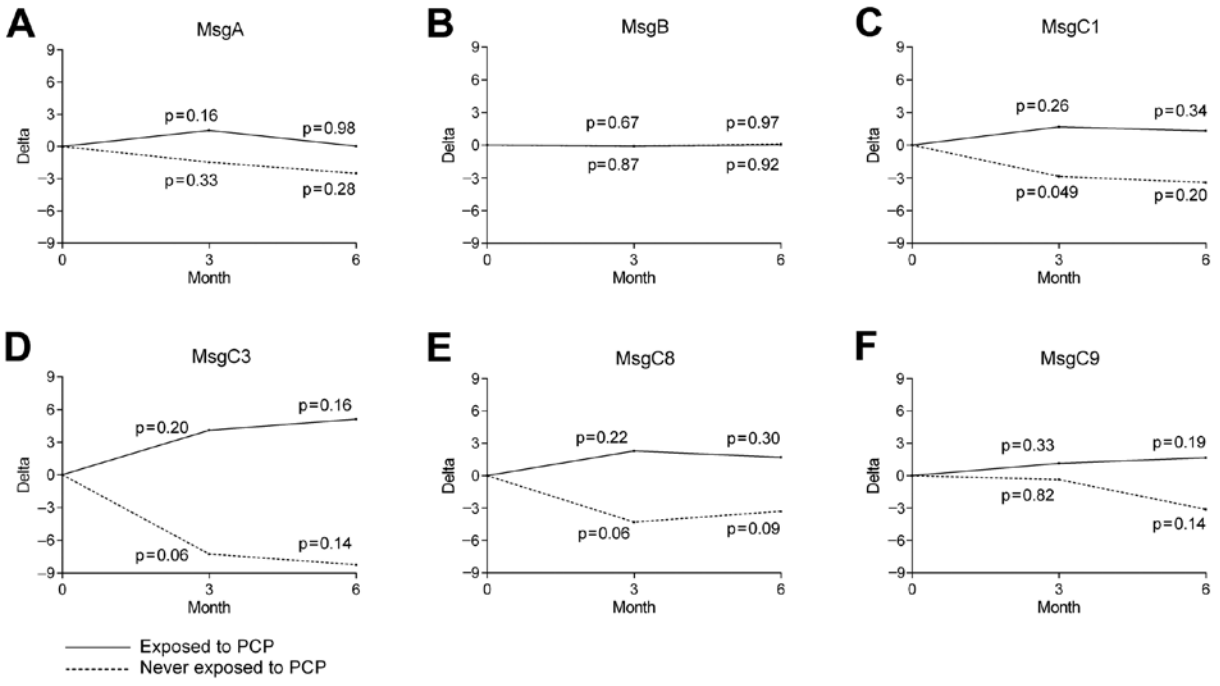

Figure 1. Differences in antibody levels against Msg at exposure to *Pneumocystis jirovecii* pneumonia (PCP) or baseline and 3 and 6 months later within groups of health care workers exposed and never exposed to PCP, San Francisco General Hospital, San Francisco, California, USA, 2007–2009. A) MsgA. B) MsgB. C) MsgC1. D) MsgC3. E) MsgC8. F) MsgC9. Msg, major surface glycoprotein.

to a PCP-infected patient is also associated with changes in antibody levels against MsgC variants over time. In our multivariate analysis, antibody levels to MsgC1 and MsgC8 were significantly higher for clinical staff than for nonclinical staff overall. Antibody levels to MsgC1, measured 3 months after baseline, had markedly declined in health care workers who were never exposed to PCP-infected patients, whereas those who were exposed to a PCP-infected patient showed no significant change in antibody levels 3 or 6 months after exposure. Significant differences were observed when we compared the change in antibody levels of all 4 MsgC variants for staff who were and were not exposed to PCP-infected patients. No significant associations were found between health care worker occupation and antibody levels to MsgA or MsgB. Also, no significant changes were found in antibody levels against MsgA or MsgB between the PCP-exposed and never PCP-exposed groups.

The carboxy terminus, or C fragment, is the most conserved region of the Msg (*25*), and IgG against MsgC1 and MsgC8 were significantly associated with clinical occupation overall. These findings are consistent with the results of our previous cross-sectional study, which found that MsgC1, but not MsgA or MsgB, antibody levels were significantly higher in clinical staff than in nonclinical staff (*23*). The current study also extends these findings to MsgC8, a variant that was not studied in the prior cross-sectional study. Previous studies involving HIV-infected patients with and without PCP infection showed that HIV-infected patients who also had PCP elicited greater responses against MsgC than did HIV-infected patients who did not have PCP (*26*). Djawe et al. found that PCP-infected patients had significantly higher serum antibody levels against MsgC1 at hospital admission, at 1–2 weeks, at 3–4 weeks, and at 5–6 weeks than did patients who had other types of pneumonia (*27*). Another study showed that patients who died of PCP had higher levels of MsgC8 than did those who died of other illnesses (*28*). A prior episode of PCP has also been shown to be a predictor of higher antibody levels against MsgC variants (*26*). These studies indicate that MsgC variants elicit significant host immune responses and antibodies against MsgC may serve as markers of infection in PCP-infected patients. Antibodies against MsgC may also be possible markers of exposure to and colonization with *P. jirovecii* in immunocompetent persons.

We observed no significant changes in antibody levels 3 or 6 months after exposure for health care workers who were exposed to PCP-infected patients. However, antibody levels against MsgC variants declined markedly in the never PCP-exposed group. When we compared the exposed and never exposed groups, we found a significant difference in mean change in antibody levels after 3 months against MsgC1, after 3 and 6 months against MsgC3, after

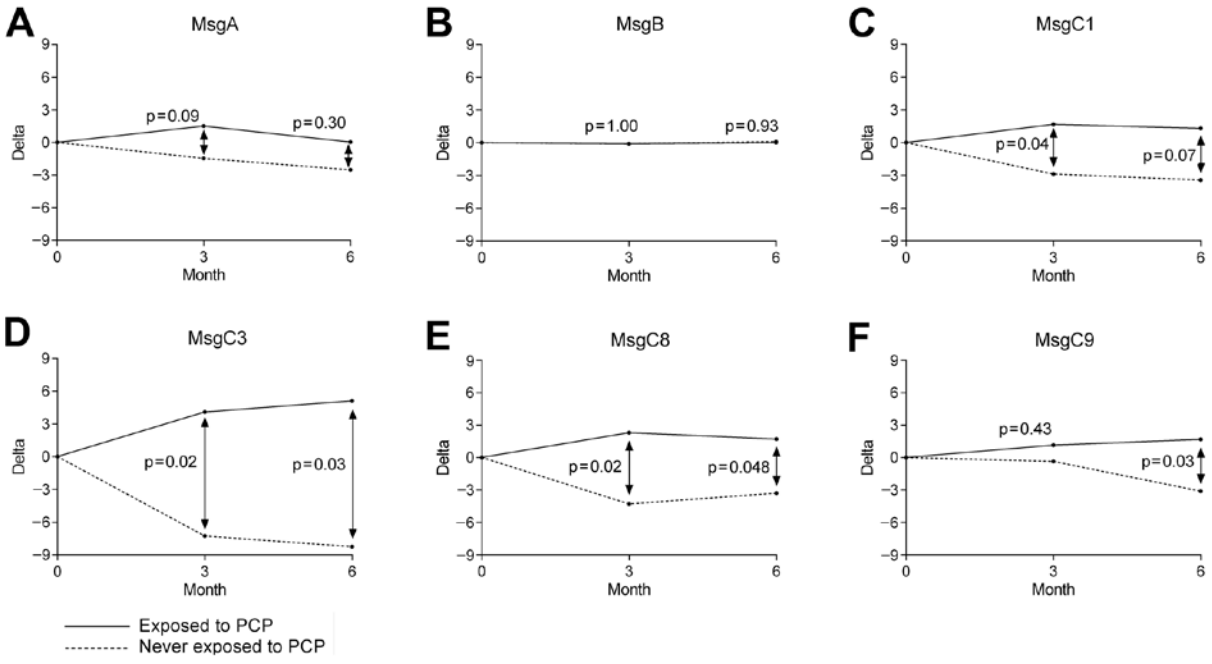

Figure 2. Differences in antibody levels against Msg at exposure to *Pneumocystis jirovecii* pneumonia (PCP) or baseline and 3 and 6 months later between groups of health care workers exposed and never exposed to PCP, San Francisco General Hospital, San Francisco, California, USA, 2007–2009. A) MsgA. B) MsgB. C) MsgC1. D) MsgC3. E) MsgC8. F) MsgC9. Msg, major surface glycoprotein.

3 and 6 months against MsgC8, and after 6 months against MsgC9. Approximately 90% of the health care workers in the exposed group were repeatedly exposed to PCP-infected patients during the 6-month period. The lack of change in antibody levels against MsgC in the exposed group suggests that health care workers who are repeatedly exposed to *P. jirovecii* continue to mount an immune response to the organism. Participants in the never-exposed group may experience a gradual decline in antibody levels because of lack of exposure to *P. jirovecii*. These data support the theory that MsgC may be a marker of *Pneumocystis* exposure and colonization in persons who have contact with PCP-infected patients.

Our study had limitations, however. One limitation was that serum specimens were collected only every 3 months, which made it difficult to correlate serum antibody levels with specific periods of patient contact. Although no significant changes were observed in health care workers 3 or 6 months after exposure to a PCP patient, significant changes that occurred 3–4 or 5–6 weeks after exposure could have been missed. Future studies that collect serum specimens more frequently after exposure are needed to accurately measure the association between exposure to a PCP-infected patient and antibody development against *P. jirovecii*. Also, given the absence of published data to inform our study design, we decided at the start of this study to enroll a large number of representative health care workers who might be exposed to HIV-infected or PCP inpatients to explore whether antibody levels against Msg differed between clinical and nonclinical staff and between PCP-exposed and never PCP-exposed groups. Future studies that focus on collecting serial serum specimens from health care workers before and after PCP patient contact will further our understanding of *P. jirovecii* antibody production in healthy human adults. A second limitation was that questions related to *P. jirovecii* exposure on initial and follow-up questionnaires captured only information about the last contact with PCP-infected patients. The follow-up questionnaires were administered every 4–8 weeks, but the question about exposure accounted for contact that occurred only within the past 4 weeks. Thus, the precise time of an exposure was ambiguous. Future studies are needed that more accurately measure the interval, duration, and frequency of exposure to a PCP-infected patient.

Results from this study demonstrate that *P. jirovecii* elicits immune responses in health care workers who are exposed to PCP-infected patients and support the theory that *P. jirovecii* is transmitted from person to person in the hospital setting. Higher antibody levels seen over time in clinical staff than in nonclinical staff suggest that clinical staff, who are exposed to PCP-infected patients, may become subclinically infected with *P. jirovecii* and mount an immune response. Because animal studies have shown

that immunocompetent mice exposed to PCP-infected mice can be carriers of *Pneumocystis* spp. and then transmit and infect immunocompromised mice, subclinically infected health care workers may thus be capable of transmitting the organism to immunocompromised patients. Future studies comparing Msg genetic sequences isolated from PCP-infected patients and the health care workers who care for them will further our understanding of *Pneumocystis* transmission. If the results from future studies support the theory of patient-to-provider transmission, respiratory precautions for PCP-infected patients may be necessary to prevent nosocomial transmission of *P. jirovecii*.

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Ms Fong is a study coordinator at San Francisco General Hospital and the University of California, San Francisco. Her research interests include immunology of *P. jirovecii*.

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Cryptococcus gattii Infections in Multiple States Outside the US Pacific Northwest

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Release date: September 19, 2013; Expiration date: September 19, 2014

Learning Objectives

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

- Distinguish characteristics of *Cryptococcus gattii* infection
- Analyze the clinical presentation of *C. gattii* infection in the United States
- Evaluate the management of *C. gattii* infection
- Distinguish outcomes of *C. gattii* infection.

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Clonal VGII subtypes (outbreak strains) of *Cryptococcus gattii* have caused an outbreak in the US Pacific Northwest since 2004. Outbreak-associated infections occur equally in male and female patients (median age 56 years) and usually cause pulmonary disease in persons with underlying medical conditions. Since 2009, a total of 25 *C. gattii* infections, 23 (92%) caused by non–outbreak strain *C. gattii*, have been reported from 8 non–Pacific Northwest states. Sixteen (64%) patients were previously healthy, and 21 (84%) were male; median age was 43 years (range 15–83 years). Ten patients who provided information reported no past-year travel to areas where *C. gattii* is known to be endemic. Nineteen (76%) patients had central nervous system infections; 6 (24%) died. *C. gattii* infection in persons without exposure to known disease-endemic areas suggests possible endemicity in the United States outside the outbreak-affected region; these infections appear to differ in clinical and demographic characteristics from outbreak-associated *C. gattii*. Clinicians outside the outbreak-affected areas should be aware of locally acquired *C. gattii* infection and its varied signs and symptoms.

Cryptococcus gattii, a fungal pathogen found in the environment, is associated with soil and decaying organic debris. Infection in humans results from inhalation of spores from the environment and typically causes pneumonia or meningitis (*1*,*2*); the incubation period is thought to be 2–13 months, although it may be longer (*3*,*4*). Unlike the related species *C. neoformans*, which is distributed globally and is a common opportunistic infection in HIV-infected or severely immunocompromised persons, *C. gattii* typically affects patients without HIV infection (*1*,*2*,*5*–*8*), and its environmental distribution is thought to be more limited (*9*–*11*). *C. gattii* infection is typically considered more difficult to treat than *C. neoformans* infection and requires longer and more aggressive treatment (*1*,*7*,*8*,*12*,*13*).

Before 1999, clinical isolates of *C. gattii* were rare in North America; a small number of cases were reported, mostly in southern California and in Hawaii (*9*,*10*,*14*,*15*). However, since 2004, an outbreak of *C. gattii* cryptococcosis has been ongoing in British Columbia, Canada, and the US Pacific Northwest states of Washington and Oregon (*2*,*5*). Approximately 100 *C. gattii* cases have been reported from Washington and Oregon. The US Pacific Northwest outbreak is characterized by infection with 3 clonal *C. gattii* strains (VGIIa, VGIIb, and VGIIc), 2 of which are uncommon outside this region and 1 (VGIIc) that is unique to the region (*16*). Previously reported cases of *C. gattii* infection were in otherwise healthy patients who had severe central nervous system (CNS) disease (*1*). In contrast, most patients associated with the US Pacific Northwest outbreak have had respiratory symptoms and preexisting immunocompromising or other serious underlying medical conditions before becoming infected with *C. gattii* (*2*).

The outbreak in the US Pacific Northwest has increased interest in *C. gattii* among public health authorities and the US health community and resulted in efforts by the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA) to collect surveillance data on *C. gattii* infections from states outside that region. Whether these reported cases are an effect of the US Pacific Northwest outbreak on other areas of the United States and the implications for clinical care and broader surveillance have not been addressed. We summarize *C. gattii* cases reported to CDC from non–US Pacific Northwest states and discuss their implications.

Methods

A case was defined as a culture-confirmed *C. gattii* infection in a resident of the United States outside of Oregon or Washington who had no known recent travel history (within 1 year) to these states and had illness onset after January 1, 2009. Cases were reported to CDC by state health departments or physicians who were treating patients with suspected *C. gattii* infection (typically because the patient was not infected with HIV). Isolates were sent to CDC for confirmation; isolates were plated on differential canavanine-glycine-bromothymol blue medium (*17*), and molecular type and subtype were identified by using multilocus sequence typing at 7 loci (*18*). Neighbor-joining trees were developed by using the multilocus sequence typing results and MEGA 4.0 software (www.megasoftware.net). State and local health department staff and treating physicians completed standardized case report forms with demographic and clinical information on case-patients and submitted these reports to CDC. Sites of infection were determined as follows: for pulmonary, blood, cerebrospinal fluid (CSF), or tissue specimens, culture was considered evidence of infection at that body site. Histopathologic examination results demonstrating *Cryptococcus* yeasts in tissue also was considered evidence of infection at the tissue site. Cryptococcal antigen in the CSF was considered evidence of CNS infection; however, serum cryptococcal antigen in the absence of a bloodstream cryptococcal isolate was not considered evidence of a bloodstream infection.

Results

Twenty-five cases for which descriptive data were available were identified in Alabama (1 case), California (13), Florida (1), Georgia (5), Hawaii (1), Michigan (1), Montana (1), and New Mexico (2). Some cases have been published as individual case reports (*2*,*19*–*21*). Twenty-one (84%) patients were male; median age was 43 years (range 15–83 years) (Table 1, Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0441-T1.htm). All patients had illness onset during January 2009–May 2012.

All patients except 1 were symptomatic. The single case in an asymptomatic patient was diagnosed by a chest radiograph during a post-trauma hospitalization; the radiograph showed upper lobe abnormalities. Four patients died before diagnosis; among the 21 patients surviving to diagnosis, the median time from symptom onset to diagnosis was 32 days (range 2–263 days). The most common symptoms were headache (67%), blurred vision (62%), and nausea (46%) (Table 1, Appendix). Leukocyte counts for 17 patients at diagnosis ranged from 6,400–20,700/mm^3 (median 11,700); CD4 counts, available for 4 patients, ranged from 92 cells/mm^3 to 838 cells/mm^3 (median 595 cells/mm^3). Twenty-three (92%) patients were hospitalized for a median of 23 days (range 1–88 days); 10 (48%) of 21 patients with data required admission to the intensive care unit. Results of lung imaging were abnormal for 19 (83%) of 23 patients with images; 14 (61%) had documented lung cryptococcomas. Results of head imaging were abnormal in 15 (75%) of 20 patients with images; 10 (50%) patients had documented brain cryptococcomas. Nineteen (76%) patients had culture, histopathologic, or serologic evidence of CNS infection, either alone or with pulmonary, blood, or tissue infections (Table 1, Appendix). Nine (36%) patients had culture or histopathologic evidence of pulmonary infection; 5 had isolated pulmonary infections. For 4 patients, *C. gattii* was isolated from blood samples.

Sixteen (64%) patients were otherwise healthy at diagnosis (Table 1, Appendix). Five (20%) patients had immunocompromising conditions (Table 1, Appendix): pulmonary sarcoidosis (CD4 count of 92 cells/mm^3), diabetes and liver transplant, an unspecified immunocompromising condition, active lung cancer (patient receiving chemotherapy), and congenital hyper-IgE syndrome (Job syndrome). Four (16%) additional patients had nonimmunocompromising underlying disease at diagnosis. For the 12 patients with documented HIV test results, all results were negative.

Six patients (ages 18, 36, 39, 56, 68, and 82 years) died, all from their *C. gattii* infections (Table 2, Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0441-T2.htm). Three were previously healthy, 1 had multiple nonimmunocompromising underlying medical conditions, and 2 were immunocompromised. One of the 3 previously healthy patients (18-year-old woman) had a fulminant course of illness that led to death 2 weeks after onset. This patient sought treatment at a community hospital with headache, fever, and flat affect; she received ceftriaxone for a urinary tract infection and was hospitalized for suspected pseudoseizures. On day 4 of her hospitalization, she became lethargic and had a low-grade fever (100.8°F). A lumbar puncture (opening pressure >60 mm H_2O) showed evidence of yeast; she was given liposomal amphotericin B and 5-flucytosine and was transferred to a referral hospital, where she received a lumbar puncture to drain CSF and endotracheal intubation for mechanical ventilation in the intensive care unit. Despite intensive supportive care, she died 2 days later of brain stem herniation caused by persistent elevated intracranial pressure (ICP).

In addition to this patient, 4 patients died before (or on the day of) their diagnoses, and 1 died 263 days after diagnosis (from shunt blockage). Three of the 4 who died before diagnosis had bloodstream infections. Three of the 4 female patients died; female sex (relative risk [RR] 5.3, 95% CI 1.6–17.3; p = 0.03) and having cryptococci isolated from blood (RR 5.3, 95% CI 1.6–17.3; p = 0.03) were significantly associated with increased risk for death. Death was not associated with other clinical or demographic variables.

Of the 21 patients surviving to diagnosis, treatment data were available for 19 (90%). All 17 who survived to diagnosis with bloodstream or CNS infections were initially given amphotericin B or liposomal amphotericin B; 16 also were given 5-flucytosine or fluconazole, and 1 was given amphotericin B alone. Of the 4 with isolated pulmonary infections who survived to diagnosis, 1 was given fluconazole alone, and 1 received amphotericin B and flucytosine; for 2, no treatment data were available.

Seven patients were given corticosteroids to treat their infection. One patient was given interferon. Twelve (57%) of 21 patients surviving to diagnosis had diagnostic or interventional surgeries, including 6 who needed a ventriculoperitoneal shunt or lumbar drain for refractory elevated ICP, 4 who needed brain or lung biopsies, 1 requiring placement of a chest tube for pleural effusion, and 1 requiring leg surgery at the infection site (*21*). Hydrocephalus developed in 4 (22%) of 18 patients with data, and cranial nerve palsies developed in 5 (38%) of 13 with data.

C. gattii isolates from patients were typed as VGI (11 [44%]), VGIII (11 [44%]), VGII (non–outbreak strain; 1 [4%]), and VGIIb (2 [8%]) (Table 3). Fifteen sequence types were represented among the 25 isolates, indicating a high degree of genetic diversity (Figure). One of the 2 VGIIb isolates was from an immunocompromised female patient from California with unknown travel history; the other was from an immunocompetent man from Florida with a leg infection and meningitis who reported no out-of-state travel for the past 20 years (*21*) (Table 1, Appendix; Table 3). Of the 13 California patients (reported from northern and southern California), 9 had unknown travel history, 2 had traveled to Mexico, and 2 had traveled to a central or eastern state in the year before their illnesses (Table 3). Ten patients (excluding the California patients) reported no exposure to known disease-endemic areas for at least the past year; 7 patients (1 from Alabama, 3 from Georgia, the Florida patient, the Montana patient, and 1 of the 2 New Mexico patients) reported no out-of-state travel for at least the past year (Table 3); 2 of these patients (New

Mexico and Montana) were incarcerated, both for <1 year, in their home states before their illness onset. Eight had outdoor exposures involving construction or gardening during the year before their illness.

Discussion

We describe severe *C. gattii* infections in the United States, many probably acquired in states outside the outbreak-affected Pacific Northwest. Most of these cases differed clinically and genetically from outbreak strains of *C. gattii* that are causing disease in the US Pacific Northwest, and they are unlikely to be associated with the outbreak. Ten patients with travel history had not traveled to areas where *C. gattii* is known to be endemic for at least 1 year before illness onset, suggesting that *C. gattii* may be endemic in some areas of the United States outside the Pacific Northwest, and infections might be going unrecognized in these areas. California is increasingly recognized as an area where *C. gattii* is endemic, and the 13 patients from California could have acquired their infections locally or, perhaps in the case of the California and New Mexico patients with travel to Mexico, in Mexico. The high case-fatality rate indicates a serious public health issue, the scope of which is unknown.

Most patients in this report were otherwise healthy men infected with *C. gattii* molecular types VGI or VGIII who had CNS disease and positive CSF cultures; fewer patients had positive pulmonary cultures or respiratory symptoms. Many patients had visual disturbances, cranial nerve palsies, and hydrocephalus when seeking treatment or that developed during treatment. Several patients required surgical interventions to mediate persistent elevated ICP. Several patients were treated with corticosteroids, possibly to manage their elevated ICP; clinical treatment guidelines for cryptococcosis recommend judicious use of therapeutic corticosteroids under specific circumstances (*13*). Cryptococcomas in the lungs and brain were common among patients in this report; these masses are more common among patients with *C. gattii* than *C. neoformans* infection (*1*). These clinical and demographic characteristics, including the disproportionately high number of infected male patients, are similar to those reported for *C. gattii* infections from other *C. gattii*–endemic areas of the world, such as Australia and Papua New Guinea, where infections are caused primarily by molecular type VGI (*1*,*2*,*5*–*8*,*22*). Most patients in our report received appropriate initial antifungal treatment; of the 2 who died after diagnosis and initiation of treatment, 1 who died 4 days after diagnosis might have received the diagnosis too late for treatment to be effective, and the other died of infection-related causes but not directly from infection. However, 4 patients died before or at diagnosis (and thus before treatment could begin). Three of these 4 had prolonged illness before diagnoses, indicating a likely window of opportunity for earlier diagnosis and treatment that might have led to improved outcomes. Information clearly is needed to determine where in the United States *C. gattii* infections pose a public health concern and where clinicians need to maintain an elevated index of suspicion for *C. gattii* infection, particularly for otherwise healthy persons who have signs and symptoms of CNS disease, in whom

Table 3. Genotype of *Cryptococcus gattii* infection and known travel history of infected patients from outside the US Pacific Northwest, 2009–2013

State	Genotype	Known travel history
Alabama	VGI	No travel for many years; decades before, lived in Hawaii, Australia, and Asia
California	VGI	Unknown
California	VGI	Past-year travel to St. Louis, Missouri
California	VGI	Unknown
California	VGI	Unknown
California	VGI	Past-year travel to Betheseda, Maryland
California	VGIII	Past-year travel to Mexico
California	VGIII	Unknown
California	VGIII	Unknown
California	VGIII	Unknown
California	VGIII	Unknown
California	VGIII	Past-year travel to Mexico
California	VGIII	Unknown
California	VGIIb	Unknown
Florida	VGIIb	No travel outside of Florida for 20 years (*21*)
Georgia	VGI	No past-year travel
Georgia	VGI	No past-year travel
Georgia	VGI	Unknown
Georgia	VGIII	No travel for at least 2 years
Georgia	VGIII	Past-year travel to North Dakota; travel 5 years before illness to Montana
Hawaii	VGII (not a/b/c)	Past-year travel to Grand Canyon, Arizona, and Las Vegas, Nevada
Michigan	VGIII	Past-year travel to Albuquerque, New Mexico, and Denver, Colorado
Montana	VGI	Florida travel 3 years before illness; incarcerated in Montana for 7 months before illness onset
New Mexico	VGI	Frequent travel to Mexico, including during year before illness
New Mexico	VGIII	Never outside of New Mexico; incarcerated in New Mexico for 2 months before illness (*20*)

cryptococcal infection might not be considered in the differential diagnoses.

The clinical signs and symptoms, host characteristics, clinical course, and molecular types of *C. gattii* infections reported here differ somewhat from those in the US Pacific Northwest outbreak. Most patients with *C. gattii* in the Pacific Northwest outbreak have underlying, often immunocompromising, medical conditions; are infected with *C. gattii* molecular types VGIIa, VGIIb, or VGIIc; and have pulmonary symptoms and positive pulmonary cultures (*2*). Approximately half of the Pacific Northwest patients are female and are a median of 56 years of age (2–13 years older than the median age of patients described here. More than half of patients in the Pacific Northwest outbreak were reported to be taking systemic oral steroids at diagnosis (*2*), a characteristic not identified among any patients in this report, and few of the patients in that outbreak reported blurred vision or required surgical interventions to mediate persistent elevated ICP during their infections (*2*).

The reasons for the clinical and demographic differences between outbreak-associated (VGIIa, VGIIb, and VGIIc) and non–outbreak-associated (other molecular types) *C. gattii* are not clear but may be related to case ascertainment differences, to the molecular type of *C. gattii* causing infection, to host differences, or a combination of these factors. Some studies have found that a patient's immune status is critical to the clinical course of cryptococcal infection (*1*,*7*,*23*); for example, HIV-uninfected persons with *C. neoformans* infections in Asia have more visual and neurologic signs and symptoms consistent with uncontrolled elevated ICP than do HIV-infected patients (*23*,*24*). Inflammation can be a defining characteristic of cryptococcal infection in immunocompetent persons (*25*–*27*) and might increase the severity of certain symptoms (*25*,*28*,*29*), which accounts for differences in clinical signs and symptoms between the largely immunosuppressed US Pacific Northwest outbreak population and the mostly healthy population reported here. Perhaps the absence (or suppression) of an intact immune response among many *C. gattii* patients in the Pacific Northwest outbreak increases risk for cryptococcal infection and attenuates its clinical severity by reducing the inflammatory response, thus reducing outcomes such as hydrocephalus and persistent elevated ICP. In contrast, an intact immune response might perversely increase the severity of certain disease symptoms among otherwise healthy *C. gattii* patients, many of whom reside outside the Pacific Northwest. However, the different strains probably also preferentially infect different patient types, perhaps because of innate requirements of different *C. gattii* strains. Day et al. and Varma et al. also found that different genetic types of *C. neoformans* infected immunocompetent and immunocompromised (HIV-infected) persons living in comparable environments and

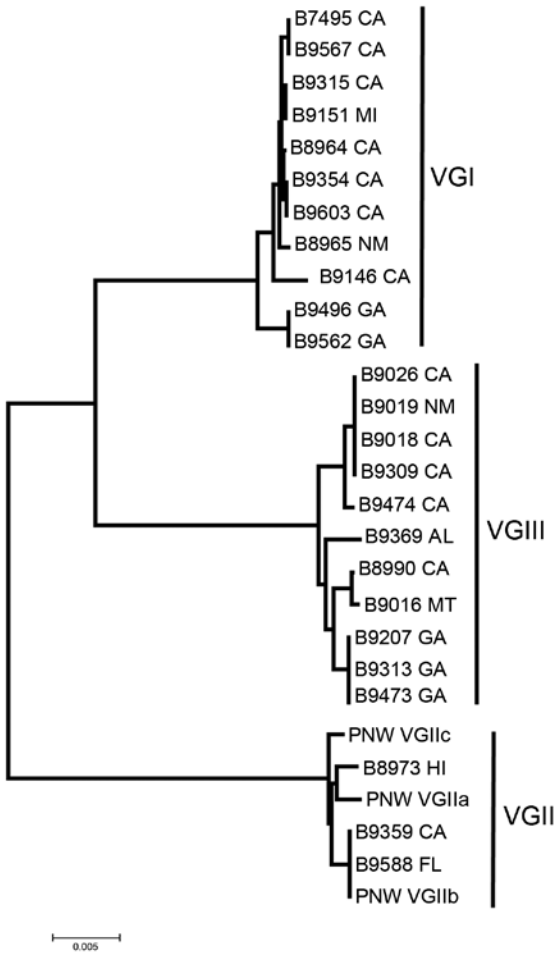

Figure. Neighbor-joining tree of US *Cryptococcus gattii* isolates from outside the US Pacific Northwest states of Washington and Oregon, 2009–2013. The tree was constructed by using multilocus sequence typing data from 7 unlinked loci. US Pacific Northwest *C. gattii* strains VGIIa, VGIIb, and VGIIc were added for reference. Scale bar indicates nucleotide substitutions per site.

circumstances (*30*,*31*); different *C. gattii* strains might also have predilections for different types of patients. Given the apparent correlation observed in the United States between molecular type of reported *C. gattii* infections and patient immune status, disentangling these characteristics might be difficult. All of the above factors—the site of infection, the strain, and the host—might be interdependent.

Our report has limitations. First, case reporting was carried out only in areas where laboratory staff, clinicians, or state health departments were aware of and interested in participating in CDC *C. gattii* surveillance. Thus, the distribution of reported *C. gattii* isolates might not represent the national distribution of *C. gattii* isolates outside the Pacific Northwest. Second, most infections in this report came from physicians specifically interested in obtaining species information for cryptococcal infections in their patients, usually

because they were HIV uninfected; thus, we cannot make inferences about the frequency of *C. gattii* infections in HIV-infected persons nor the clinical course of *C. gattii* disease in HIV-infected persons from this report. Case ascertainment among patients in this group was also likely to differ from ascertainment in the Pacific Northwest, and comparisons between patients in each group should be interpreted with care. Additionally, co-existing pulmonary disease among these patients might be underreported. Although clinicians are likely to try to rule out CNS infection in patients with pulmonary cryptococcosis, such as those in the Pacific Northwest, patients with CNS cryptococcosis might be less likely to have pulmonary sites cultured because identifying the site will not influence treatment. Finally, we might have underestimated travel-associated infections; although most *C. gattii* infections are considered to have an incubation of 2–13 months (*3*,*4*), incubations as long as 3 years have been reported (*32*). Because most *C. gattii* incubation period data are based on persons exposed in the Pacific Northwest outbreak, and the outbreak is still relatively nascent, longer incubation periods might manifest as the time since exposure to the outbreak-affected region lengthens.

Although this case series might represent an emerging public health issue in states outside the US Pacific Northwest, it also might represent previously unrecognized disease in these states. Individual clinicians submitted multiple cases for this report, which suggests hotspots of environmental colonization, hotspots of individual interest, or both. However, *C. gattii* infections have been documented, albeit rarely, in at least some of these areas of the United States: in addition to the few cases identified in southern California, where *C. gattii* previously has been thought to be endemic, and Hawaii (*9*,*10*,*14*,*15*,*33*), a small number of cases also were reported in Georgia (*19*,*33*,*34*). Unlike the clonal outbreak strains found in the Pacific Northwest, the diversity and lack of clonality among the VGI and VGIII isolates from patients reported here indicates that these pathogens most likely have been propagating and diversifying in the United States for a long time. Anecdotal information about and treatment guidelines for *C. gattii*, primarily based on cases from Australia and Papua New Guinea, suggest that *C. gattii* infections might require more aggressive or longer treatment than *C. neoformans* infections (*1*,*7*,*8*,*12*,*13*).

An increased index of suspicion for *C. gattii* infection may be warranted in some areas of the United States; recognition of species *gattii* infection and early diagnosis might improve outcomes. Species identification of cryptococcal isolates requires specialized agar; however, most clinical laboratories do not identify isolates to the species level. Routine surveillance and species and molecular type identification for *C. gattii* outside the Pacific Northwest would enable identification of the true range of infections, and shed light on where diagnostics for *C. gattii* would be most useful.

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Rickettsia slovaca Infection in Humans, Portugal

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Fifteen years after the initial detection of *Rickettsia slovaca* in ticks in Portugal, 3 autochthonous cases of *R. slovaca* infection were diagnosed in humans. All patients had an eschar on the scalp and lymphadenopathy; 2 patients had facial edema. *R. slovaca* infection was confirmed by serologic testing, culture, and PCR.

Rickettsia slovaca is a pathogenic, tick-borne, spotted fever group (SFG) rickettsiae that was initially isolated in 1968 from a *Dermacentor marginatus* tick in Slovakia. *R. slovaca* infection has been described in humans from several countries (*1*,*2*), but a laboratory-confirmed case of *R. slovaca* infection was first reported in a patient in France in 1997 (*3*). *R. slovaca* has since been reported in humans in France, Spain, Hungary, Slovakia, Bulgaria, Italy, and Germany (*4*–*7*).

The analysis of a large series of patients reporting the common clinical signs of enlarged regional lymph nodes associated with the tick bite led to the names that have been used to designate this rickettsial disease, tick-borne lymphadenopathy (TIBOLA) and *Dermacentor*-borne-necrosis-erythema lymphadenopathy (*8*,*9*). Apart from Mediterranean spotted fever, TIBOLA may be among the most prevalent tick-borne rickettsioses in Europe (*4*).

In Portugal, *R. slovaca* was initially described in 1995 in *D. marginatus* ticks and later in *D. reticulatus* ticks (*10*,*11*) but has not been identified in humans. We report 3 laboratory-confirmed cases of *R. slovaca* infection in human patients in Portugal.

The Study

During October 2010–May 2012, three Caucasian women who sought care at the emergency department of University Hospitals of Coimbra (HUC), Coimbra, Portugal, were admitted with clinical signs and symptoms compatible with a rickettsiosis. The patients were residents of rural areas of the Coimbra district, and all were at risk for tick exposure through fieldwork or direct contact with domestic animals (Table). Two patients reported that they had removed a tick from the scalp.

Physical examination showed a single inoculation eschar surrounded by an erythematous halo on the scalps of all 3 patients; 1 patient later had alopecia develop at the tick bite site (Table; Figure 1). Two patients also had painful cervical and occipital lymphoadenopathies, accompanied by unilateral or bilateral peri-orbital edema (Table; Figure 2). Fever (37.8°C) and a maculopapular rash in the trunk and upper limbs were each visible and reported in 1 patient.

Laboratory testing showed a slight increase of C-reactive protein in 2 patients and a mild thrombocytopenia in 1, but other results were within normal limits. The patients were treated with doxycycline (200 mg/d) for 7 days with progressive resolution of the clinical signs (e.g., edema, rash).

To confirm the diagnosis of *R. slovaca* infection, serum samples and skin biopsy specimens collected at different times of infection from all patients (Table) were sent to the Portuguese reference laboratory for rickettsioses. Serologic response was analyzed by in-house immunofluorescence assay using *R. slovaca* PoTi443 strain as antigen, as described (*12*). Seroconversion in 2 consecutive samples was demonstrated in patients 1 and 2 by the appearance or increasing levels of IgM and IgG against rickettsiae (Table). Patient 3 had only 1 acute serum sample; test results showed titers for IgM were 32 and for IgG were 64.

Molecular detection of rickettsial DNA on eschar biopsies was performed by PCR, as described (*13*). Briefly, DNA was extracted from each skin biopsy sample by using a DNeasy Tissue Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Nested PCR amplification targeting *omp*A (190-kDa protein) and citrate synthase (*glt*A) fragment genes of *Rickettsia* spp. and sequencing of positive products were done as described (*13*). The sequences were edited by using Lasergene software (DNASTAR, Madison, WI, USA). BLAST analysis (http://blast.ncbi.nlm.nih.gov) showed 100% homology with *glt*A (382/382 bp) and *omp*A (323/323 bp) of *R. slovaca* isolate PoTi443 (GenBank accession nos. HM149281 and HM149286), which was detected in *D. marginatus* ticks in Portugal (*11*).

Skin samples from patients 1 and 3 were used for rickettsial isolation attempts by using Vero E6 cell line and shell-vial technique as described (*14*). (The sample from patient 2 was too small to use for both tests.) After 8 days' incubation at 32°C, growth of *Rickettsia* spp. was detected and visualized by Gimenez staining and imunofluorescence assay. Positive culture was confirmed for patient 1;

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Table. Epidemiologic, clinical and microbiologic characteristics of 3 patients infected with *Rickettsia slovaca*, Portugal

Characteristic	Patient 1	Patient 2	Patient 3
Epidemiologic			
Age, y/sex	50/F	53/F	30/F
Date of illness onset	2010 Oct	2012 Apr	2012 May
Type of residence	Rural	Rural	Rural
At-risk activity	Field worker	Field worker, contact with domestic animals	Contact with dogs
Reported tick bite	Yes	Yes	NA
Clinical characteristics			
Incubation time, d*†	4	7	NA
Fever	Yes, 37.8°C	No	No
Eschar on scalp	Yes	Yes	Yes
Rash	No	Yes, maculopapular	No
Painful cervical/occipital lymphadenopathy	Yes	No	Yes
Facial edema	Yes	Yes	No
Headache	Yes	No	No
Localized alopecia	No	Yes	No
Laboratory diagnostics			
Antibodies against *R. slovaca* by IFA‡			
Sample 1, titer IgM/IgG	Negative	32/64	32/64
Sample 2, titer IgM/IgG	32/128	1,024/1,024	ND
Culture from eschar	Positive	ND	Contaminated
PCR from eschar	Positive	Positive	Positive

*All patients were treated with doxycycline. NA, not available; ND, not done; IFA, immunofluorescence assay.
†From tick bite to symptom onset.
‡Positive cutoff values, IgM = 32; IgG = 128.

however, the culture from patient 3 was contaminated. The *Rickettsia* spp. isolate was characterized by PCR and sequencing as described above for molecular detection. This analysis provided definitive confirmation of the isolate as *R. slovaca.*

Conclusions

We report 3 confirmed cases of TIBOLA in Portuguese patients, an indication of the emergence of this rickettsial disease in Portugal. The patients were all women, which is in accordance with previous findings of a higher risk for infection for women and children (*4*). Our patients also each showed a tick bite on the scalp associated with the enlargement of lymph nodes, as described in other clinical reports (*3*,*4*–*7*,*9*,*15*).

Two (67%) of the patients we describe showed facial edema, which is notably higher than Parola et al. reported in his series of patients, where facial edema occurred in 6 (19%) of 49 patients (*15*). Although our number of patients was very small, this sign, associated with the eschar on the scalps of patients 1 and 2, is what led clinicians to further investigate which species of *Rickettsia* was involved in these infections. One of the patients showed residual alopecia, but no patients reported persistent fatigue; these have been described in other patients as frequent complaints in the convalescent stage of disease (*3*,*5*). Low-grade fever (37.8°C) and maculopapular rash each occurred in 1 patient, similar to rates in previous reports for fever (12%–67%) and rash (14%–23%) (*3*,*4*,*15*).

Aside from the typical manifestations of TIBOLA in these patients, isolation and PCR detection followed by genetic characterization of isolates were essential to confirm *R. slovaca* infection. Although the patients showed detectable antibodies against *R. slovaca*, diagnosis on the basis of serologic results only does not distinguish among various SFG rickettsiae, and in Portugal, different Rickettsia spp. can circulate during the same time of year (*13*,*14*). The onset of symptoms (media incubation time 5.5 days) in these patients was in October, April, and May, timing that is associated with seasonal activity of *Dermacentor* spp. ticks. Prevalence rates of *R. slovaca* in these ticks in Europe range from 21% in Hungary to 40.6% in Spain and

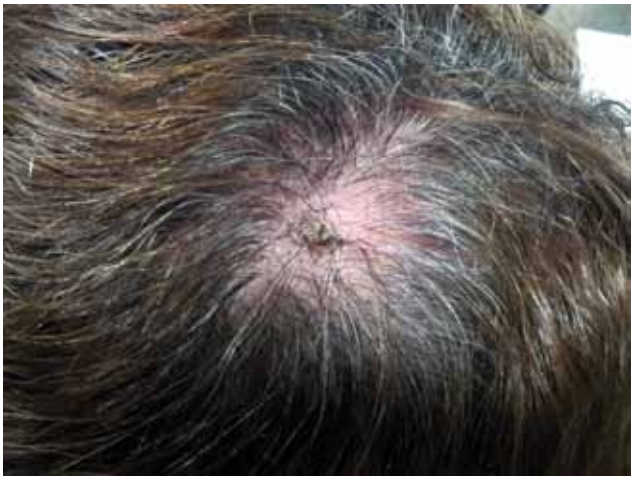

Figure 1. Inoculation eschar surrounded by an erythematous halo at the site of a tick bite on the scalp of a female patient in Portugal. Tick-borne lymphoadenopathy caused by *Rickettsia slovaca* infection was later confirmed.

Figure 2. Left peri-orbital edema in a female patient in Portugal. Tick-borne lymphoadenopathy caused by *Rickettsia slovaca* infection was later confirmed.

41.5% in Portugal (*11*). Based on the similar prevalence of *R. slovaca* in ticks in Portugal and Spain, and in comparison with the large number of patients in Spain with *R. slovaca* infection, it is possible that cases of *R. slovaca* infection in Portugal are not being recognized by clinicians or are being misdiagnosed as Mediterranean spotted fever.

Although *R. slovaca* is the main etiologic agent associated with TIBOLA, recent studies have indicated that patients with the same characteristic clinical signs may be infected with other *Rickettsia* species, such as *R. rioja* or *R. raoultii* (*4*,*11*), which are also transmitted by *Dermacentor* spp. ticks. Oteo et al. reported that, in Spain, *R. rioja* was the causative agent for almost half of patients with TIBOLA (*4*). In Portugal, a high prevalence (58.5%) of *R. raoultii* has found in *Dermacentor* spp., but this rickettsial species has not been detected in humans (*11*). Because clinical signs can overlap in different rickettsial infections and serologic testing cannot distinguish among SFG rickettsiae, molecular characterization is essential to clarifying the epidemiology of these rickettsial infections.

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Reassortant Avian Influenza A(H5N1) Viruses with H9N2-PB1 Gene in Poultry, Bangladesh

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Bangladesh has reported a high number of outbreaks of highly pathogenic avian influenza (HPAI) (H5N1) in poultry. We identified a natural reassortant HPAI (H5N1) virus containing a H9N2-PB1 gene in poultry in Bangladesh. Our findings highlight the risks for prolonged co-circulation of avian influenza viruses and the need to monitor their evolution.

Bangladesh has one of the highest reported number of outbreaks of highly pathogenic avian influenza (HPAI) (H5N1) in poultry (*1*). As of May 26, 2013, a total of 548 outbreaks of HPAI (H5N1) have been reported (*1*); these outbreaks have resulted in serious economic repercussions in the poultry sector in this country. Furthermore, 7 cases of human infection with HPAI (H5N1) were confirmed, most recently a fatal case in a 2-year-old child in April 2013 (*2*).

In addition to the HPAI (H5N1) virus, the H9N2 subtype is widely circulating in poultry in Bangladesh, which raises concerns about the possible implications of the extensive co-circulation of these viruses (*3*,*4*). Their coexistence in the same susceptible population is likely to generate appropriate conditions for the emergence of novel reassortant variants, with unknown epizootic and zoonotic potential. We characterized the complete genome of 18 HPAI (H5N1) virus strains from Bangladesh and report the identification and characterization of a novel natural reassortant HPAI (H5N1) virus that contains an H9N2-PB1 gene in poultry.

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The Study

A total of 18 tracheal and 1 cloacal samples were collected from chickens in 14 layer farms (15 samples) and 2 live-bird markets (4 samples) in 13 districts of Bangladesh during December 2011–April 2012. The samples were submitted by the Department of Livestock Services Dhaka to the World Organisation for Animal Health/United Nations Food and Agriculture Organization Reference laboratory for Avian Influenza in Italy for confirmatory diagnosis and genetic analysis. All samples tested positive for the H5N1 subtype by real-time reverse transcription PCR (rRT-PCR) (*5*) and virus isolation (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf). Because the H7 subtype is also notifiable and the H9N2 subtype circulates in the poultry population in Bangladesh (*3*), samples were tested for these subtypes as well; results were negative. Epidemiologic information for these viruses is provided in the Table.

The complete genome of 18 HPAI (H5N1) isolates and the hemagglutinin (HA) gene of 1 virus (A/chicken/Bangladesh/12VIR-7140-9/2012) were successfully sequenced, as described (*6*). The nucleotide sequences obtained in this study have been submitted to GenBank (accession nos. KC616462–KC616606). Maximum-likelihood (ML) trees were estimated for all 8 gene segments (HA, neuraminidase [NA], nucleoprotein [NP], basic polymerase protein [PB] 1 and 2, polymerase [PA], matrix [M], and nonstructural [NS]) by using the best-fit general time reversible + I + Γ4 model of nucleotide substitution in PhyML (*7*). A bootstrap resampling process (1,000 replications) using the neighbor-joining method was used to assess the robustness of individual nodes of the phylogeny, incorporating the ML substitution model defined above.

Analysis of the topologic differences between the 8 ML phylogenetic trees revealed that an intersubtype reassortment event took place and caused replacement of the PB1 gene of 2 of the HPAI (H5N1) viruses from Bangladesh (A/chicken/Bangladesh/12VIR-7140-7/2012 and A/chicken/Bangladesh/12VIR-7140-16/2012) with the PB1 gene of an H9N2 subtype virus. In the HA gene, all the viruses from Bangladesh analyzed fell within clade 2.3.2.1 (Figure 1) and had high similarity (98.2%–99.7%) to viruses from Bangladesh and India identified in 2011 (*10*). Phylogenetic analysis of the NA, NP, PB2, PA, M, and NS genes (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0534-Techapp1.pdf) confirmed the clustering observed for the HA gene; all 18 fully sequenced

Table. Avian influenza A(H5N1) virus strains analyzed in study of reassortant viruses in poultry, Bangladesh, December 2011–April 2012

Strain	Division, district, city	GPS coordinates, decimal degrees, N, E	Date of sample collection	Type of production
A/chicken/Bangladesh/Khulna/12VIR-7140–1/2011	Khulna, Bagerhat, Sonatala	22.477883, 89.652883	Dec 19	Layer
A/chicken/Bangladesh/Khulna/12VIR-7140–2/2012	Khulna, Meherpur	23.775000, 88.6417	Jan 2	Layer
A/chicken/Bangladesh/Dhaka/12VIR-7140–3/2012	Dhaka, Demra	23.707533, 0.471667	Jan 8	Layer (hatchery)
A/chicken/Bangladesh/Dhaka/12VIR-7140–4/2012	Dhaka, Manikgahj, Shibalaya	23.828967, 89.7854	Jan 10	Layer (hatchery)
A/chicken/Bangladesh/Dhaka12VIR-7140–5/2012	Dhaka, Narayanganj, Araihajar	23.78395, 90.6517	Jan 21	Layer (hatchery)
A/chicken/Bangladesh/Dhaka12VIR-7140–6/2012	Dhaka, Tangail	24.684433, 89.95545	Feb 14	Layer (hatchery)
A/chicken/Bangladesh/Rajshahi/12VIR-7140–7/2012	Rajshahi, Sirajganj, Tarash	24.363633, 89.361617	Jan 19	Layer (hatchery)
A/chicken/Bangladesh/Chittagong/12VIR-7140–8/2012	Chittagong, Feni	22.9294, 91.300933	Feb 14	Layer (hatchery)
A/chicken/Bangladesh/Dhaka/12VIR-7140–9/2012	Dhaka, Gazipur	23.89306, 90.330319	Feb 16	Layer (hatchery)
A/chicken/Bangladesh/Khulna/12VIR-7140–10/2012	Khulna, Khulna	22.708517, 89.353517	Mar 5	Layer (hatchery)
A/chicken/Bangladesh/Dhaka/12VIR-7140–11/2012	Dhaka, Dhaka, Dohar	23.6091, 90.13205	Mar 18	Layer (hatchery)
A/chicken/Bangladesh/Khulna/12VIR-7140–12/2012	Khulna, Jessore	23.182367, 89.190183	Mar 27	Layer (hatchery)
A/chicken/Bangladesh/Rangpur/12VIR-7140–13/2012	Rangpur, Nilphamari	25.91686, 88.98789	Apr 3	Layer
A/chicken/Bangladesh/Rangpur/12VIR-7140–14/2012	Rangpur, Nilphamari	25.91686, 88.98789	Apr 3	Layer
A/chicken/Bangladesh/Dhaka/12VIR-7140–15/2012	Dhaka, Rajbari, Pangsha	23.736233, 89.54385	Apr 5	Layer (hatchery)
A/chicken/Bangladesh/Dhaka/12VIR-7140–16/201	Dhaka (Kaptan Bazar), Dhaka, Dhaka	23.716765, 90.400245	Mar 13	Live-bird market
A/chicken/Bangladesh/Dhaka/12VIR-7140–17/2012	Dhaka (Anando Bazar), Rajbari, Dhaka	23.716783, 90.400053	Mar 21	Live-bird market
A/chicken/Bangladesh/Dhaka/12VIR-7140–18/2012	Dhaka (Tejgaon Bazar), Dhaka, Dhaka	23.750155, 90.383516	Mar 21	Live-bird market
A/chicken/Bangladesh/Dhaka/12VIR-7140–19/2012	Dhaka (Kaptan Bazar), Dhaka, Dhaka	23.716765, 90.400245	Mar 25	Live-bird market

viruses grouped together and with viruses from Bangladesh and India identified in 2011.

A close examination of the PB1 tree topology revealed the existence of a diversified origin of this internal gene for the viruses analyzed. In particular, the PB1 gene of 16 of the 18 viruses we characterized clustered as described for the other 7 genome segments, whereas phylogeny of A/chicken/Bangladesh/12VIR-7140-7/2012 and A/chicken/Bangladesh/12VIR-7140-16/2012 revealed that their PB1 gene derived from H9N2 subtype viruses. In particular, the PB1 gene of these 2 HPAI (H5N1) strains belongs to a cluster composed by subtype H9N2 viruses from Bangladesh and India identified during 2008–2011 and had the highest similarity (96.9%–97.1%) with the Indian strain A/chicken/Tripura/105131/2008. Genetic identity between A/chicken/Bangladesh/12VIR-7140-7/2012 and A/chicken/Bangladesh/12VIR-7140-16/2012 ranged from 98.1% for the PA gene to 99.3% for the NP gene; the 2 viruses can be distinguished from each other by a total of 37 aa differences found in the 13 viral proteins, with the PA gene possessing most (16) of these substitutions. Furthermore, distinct epidemiologic origins characterize these viruses; the A/chicken/Bangladesh/12VIR-7140-7/2012 virus was identified in a chicken farm located in Sirajganj district in January 2012, whereas the A/chicken/Bangladesh/12VIR-7140-16/2012 strain was detected 2 months later (March 2012) from organs collected in Kaptan Bazar in Dhaka (Table), which is 126 km from Sirajganj district. By evaluating these findings, we can theorize that a single reassortant event occurred and then the viruses evolved independently or that 2 independent reassortant events took place.

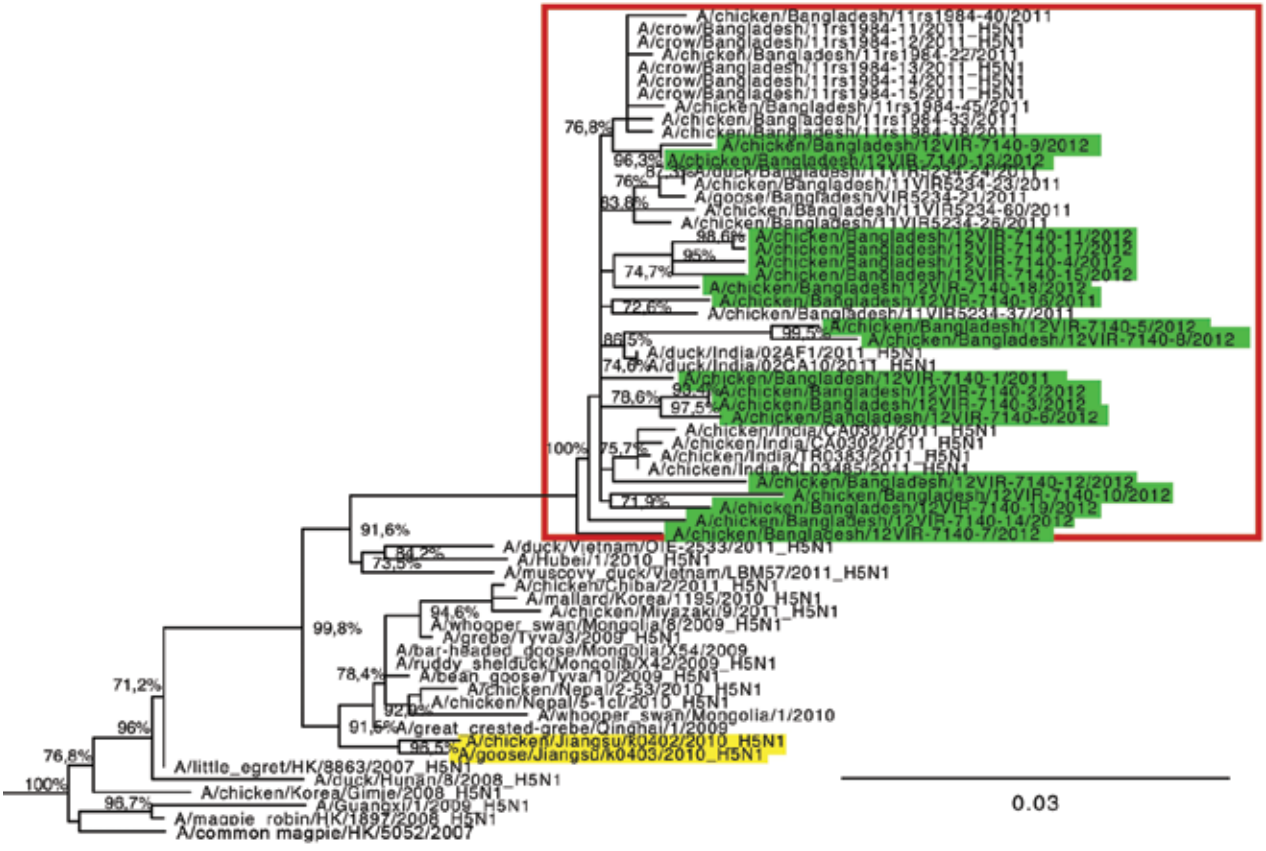

Figure 1. Partial maximum-likelihood phylogenetic tree for the hemagglutinin gene segment of avian influenza (H5N1) viruses from Bangladesh compared with other viruses within clade 2.3.2.1. Red box indicates avian influenza (H5N1) viruses from Bangladesh (2011–2012) and India (2011). Green shading indicates viruses from Bangladesh sequenced and characterized in this study; yellow shading indicates previously described subtype H5N1/H9N2 reassortant influenza viruses (*8*,*9*) or those from GenBank. Numbers at the nodes represent bootstrap values. Scale bar indicates nucleotide substitutions per site. An expanded version of this figure, showing the complete phylogenetic tree generated, is available online (wwwnc.cdc.gov/EID/article/19/10/13-0534-F1.htm).

The genetic characterization of the 2 natural reassortant viruses we identified was remarkably different from that of previously identified reassortant viruses possessing subtype H9N2 genes in an HPAI (H5N1) backbone virus identified in Asia (*8*,*9*). Our results imply that these natural reassortments occurred independently. Indeed, the phylogenic trees for the HA and PB1 genes (Figures 1, 2) demonstrate that the 2 viruses from Bangladesh can be distinguished from the H5N1/H9N2 reassortant strains identified in Tibet and China.

The pathogenicity of 3 subtype H5N1 viruses (the 2 H5N1/H9N2 7:1 reassortants and the H5N1 A/chicken/Bangladesh/12VIR-7140–12/2012 isolate) was determined by intravenous pathogenicity index in specific pathogen–free chickens. This value was 3.0 for all the viruses tested, confirming the isolates can be considered highly pathogenic, according to the World Organisation for Animal Health definition (www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf).

Conclusions

Although reassortment represents a major mechanism behind the emergence and evolution of the HPAI (H5N1) in Asia (*11*,*12*), data suggest that, in the past decade, these viruses have only sporadically provided the backbone for generation of novel intersubtype reassortants (*8*,*9*,*13*,*14*). This is surprising considering the prolonged and extensive co-circulation of subtype H5N1 and H9N2 viruses in poultry in Asia; it is unclear whether the limited evidence is because of insufficient surveillance or the low capacity of subtype H5N1 viruses to undergo intersubtype reassortment. We have documented the emergence and circulation of a novel natural H5N1/H9N2 7:1 reassortant strain in Bangladesh in 2012. This finding shows that the HPAI (H5N1) viruses circulating in this area are continuously evolving. Although it is difficult to ascertain where and when the reassortment event occurred, the identification of genetic clustering of the strains analyzed here with subtype H5N1 and H9N2 viruses identified in India confirms

intense cross-border transmission between these regions, which has resulted in a favorable epidemiologic system for influenza viruses evolution (*15*).

The emergence of these natural H5N1/H9N2 reassortant influenza viruses suggests that co-infections with viruses of different subtypes have presumably occurred in poultry, most likely a result of the persistent co-circulation of these viruses along with poor biosecurity measures. This possibility underlines the importance of providing poultry farmers and small-holder poultry

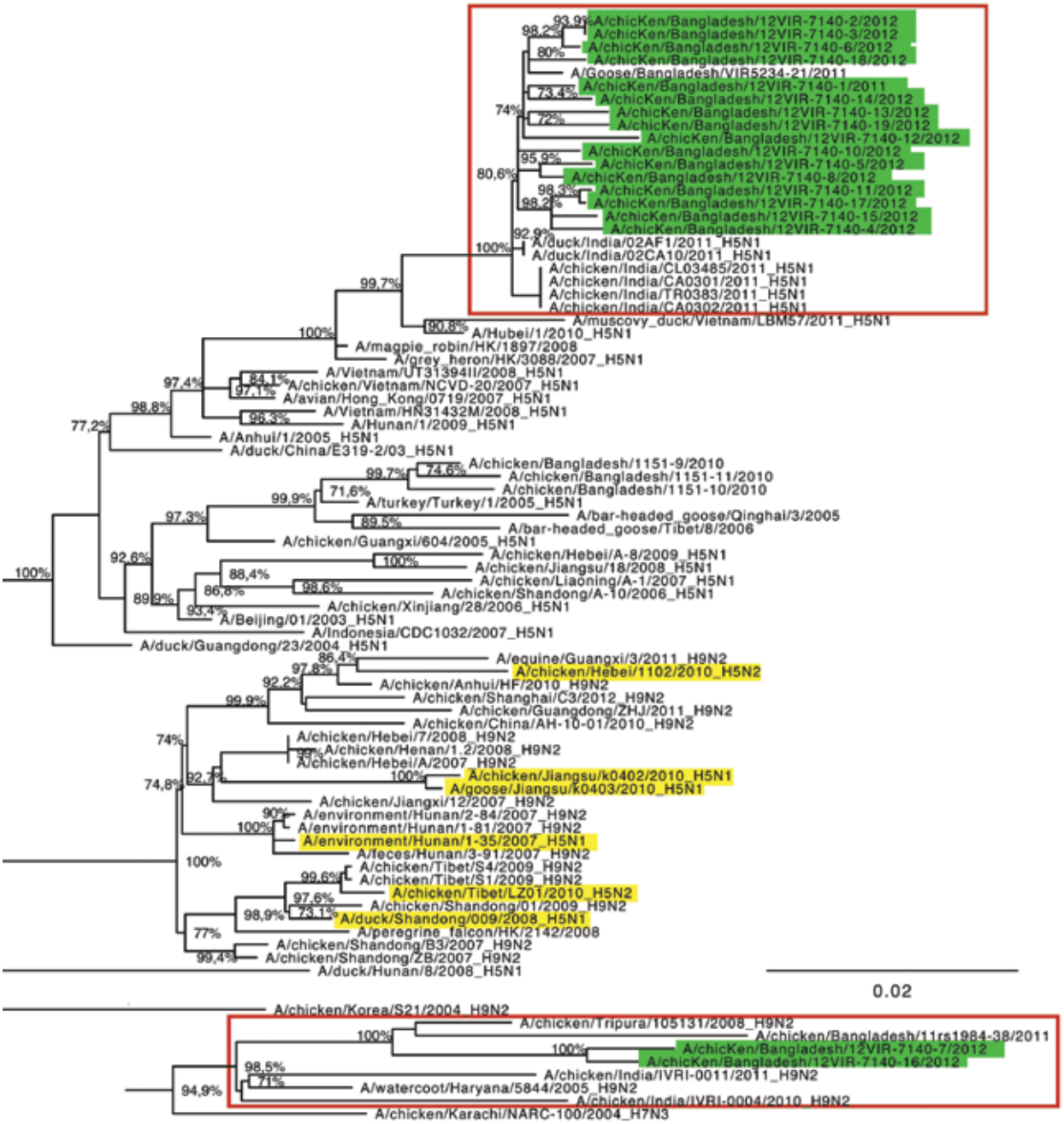

Figure 2. Partial maximum-likelihood phylogenetic tree for the basic polymerase 1 gene segment of avian influenza (H5N1) viruses from Bangladesh compared with other viruses. Red boxes indicate avian influenza (H5N1) viruses from Bangladesh (2011–2012) and India (2011) and influenza (H9N2) viruses from Bangladesh (2011) and India (2010–2011). Green indicates viruses from Bangladesh sequenced and characterized in this study; yellow indicates previously described subtype H5N1/H9N2 reassortant influenza viruses (*8*,*9*) or those from GenBank. Numbers at the nodes represent bootstrap values. Scale bar indicates nucleotide substitutions per site. An expanded version of this figure, showing the complete phylogenetic tree generated, is available online (wwwnc.cdc.gov/EID/article/19/10/13-0534-F2.htm).

producers with educational programs about appropriate control measures for avian influenza. Furthermore, constant efforts must be undertaken to continue monitoring the evolution of influenza A(H5N1) viruses in Bangladesh and bordering countries to estimate the spread of this novel variant and to trace its origin and evolution. There is evidence that the polymerase subunits have a key role in virus replication efficiency and cross-species transmission; therefore, pathogenicity and transmission studies in poultry and mammal models are essential to evaluate the potential animal and public health threat posed by these novel H5N1/H9N2 reassortant influenza viruses.

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Evolution of Influenza A Virus H7 and N9 Subtypes, Eastern Asia

Camille Lebarbenchon, Justin D. Brown, and David E. Stallknecht

Influenza A viruses are a threat to poultry and human health. We investigated evolution of influenza A virus H7 and N9 subtypes in wild and domestic birds. Influenza A(H7N9) virus probably emerged after a long silent circulation in live poultry markets in eastern Asia.

Emergence of influenza A(H7N9) virus in China raised concerns about potential virus adaptation to mammals and human-to-human transmission (*1,2*). Investigations of virus sources and vectors are needed because they will provide useful information about influenza A(H7N9) virus subtype evolution and adaptation processes. Wild waterbirds are natural hosts for influenza A viruses and are sources for introduction of virus into poultry, in which the viruses adapt and sometimes evolve toward increased virulence (H5 and H7 virus subtypes). Although H7 subtype influenza A viruses have been isolated from wild birds worldwide, the role of these hosts in emergence, maintenance, and potential intercontinental spread of influenza A(H7N9) virus has not been determined.

We analyzed molecular evolution of H7 (hemagglutinin) and N9 (neuraminidase) subtypes of avian influenza virus. The purpose of this study was to investigate the recent evolutionary history of H7 and N9 virus subtypes in eastern Asia and identify the most recent wild bird ancestor of influenza A(H7N9) virus hemagglutinin and neuraminidase.

The Study

To assess global phylogeny of influenza A virus H7 and N9 subtypes, we analyzed 715 hemagglutinin and 309 neuraminidase nucleotide sequences of viruses isolated during 1927–2012 worldwide (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0609-Techapp1.pdf). Bayesian Markov Chain Monte Carlo coalescent analyses were conducted to investigate recent evolutionary history of influenza A virus H7 and N9 subtypes in eastern Asia by using BEAST version 1.7.4 (*3,4*).

Phylogenetic analyses showed maintenance of influenza A virus H7 subtypes in wild birds in eastern Asia since 1999 (clade A) (Figure 1, panel A). More specifically, circulation has been restricted mainly to the eastern Asia flyway; most viruses isolated from wild birds were from Japan and South Korea. This local perpetuation in wild birds has favored several independent introductions of viruses into poultry in Japan, South Korea, eastern China (Jiangxi and Zhejiang Provinces), and Thailand. A genetically different virus H7 subtype lineage was detected in Europe and Asia during 2006–2012 (clade B) (Figure 1, panel B). These results suggest that ≥2 influenza A virus H7 subtypes co-circulated in eastern Asia during that period. In Japan, replacement of influenza A virus H7 subtypes that were circulating in wild ducks during 2008 (clade A) may have occurred because recent viruses isolated from ducks (2011–2012) all belong to clade B. The same pattern was observed in Thailand: influenza A virus H7 subtypes isolated in 2011 were genetically different from most viruses isolated in 2010.

This pattern suggests that the old genetic lineage of influenza A virus H7 subtypes that circulated in eastern Asia since 1999 (clade A) may have been progressively replaced by a more recent lineage (clade B). Clustering of clade B viruses with those isolated in Europe suggest that gene flow has recently occurred in Eurasia, probably as the result of waterfowl migrations and poultry trade. However, the European origin of viruses from Asia was not supported on the basis of phylogeographic analysis (online Technical Appendix).

Consistent with results of previous studies (*1,2,5,6*), our results indicate that hemagglutinin of human influenza A(H7N9) viruses belongs to clade A and is most genetically related to influenza viruses isolated from domestic ducks at live-poultry markets in Zhejiang Province, China (*7*). Our findings further indicate that hemagglutinin of human influenza virus did not evolve from the H7 HA circulating in these domestic birds but was derived from a common ancestral influenza A virus circulating in an unidentified host during 2010. The most recent common ancestral influenza A virus among A/Hangzhou/1/2013(H7N9) virus, Zhejiang domestic duck(H7N3) virus, and influenza A virus H7 subtype circulating in wild birds could be dated to 2004 (Figure 1, panel A), indicating that silent introduction and circulation of influenza A virus H7 subtypes in domestic animals might have occurred in this virus before influenza A(H7N9) virus was identified in humans (*8*).

Limited epidemiologic and genetic information about influenza A virus H7 subtype circulating in eastern China

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during 2004–2011 precludes more precise conclusions on origins of human influenza A(H7N9) virus and relatedness to influenza A virus circulating in wild birds. However, on the basis of genetic analyses of recently isolated viruses from chickens, pigeons, and the environment, maintenance and genetic reassortment of emerging influenza A(H7N9) virus might have occurred in live poultry markets in Shanghai, China (*5*,*7*).

The phylogenetic structure we observed for influenza A virus subtype N9 suggests that gene flow has occurred since 1996 among Europe, Africa, Asia, and Oceania (Figure 2; online Technical Appendix). Analyses showed circulation of influenza A virus subtype N9 (mainly H11N9 subtype) (Figure 2) in eastern Asia since 2003, and evidence of virus dispersal to Europe and Australia and reassortments with hemagglutinin of avian influenza

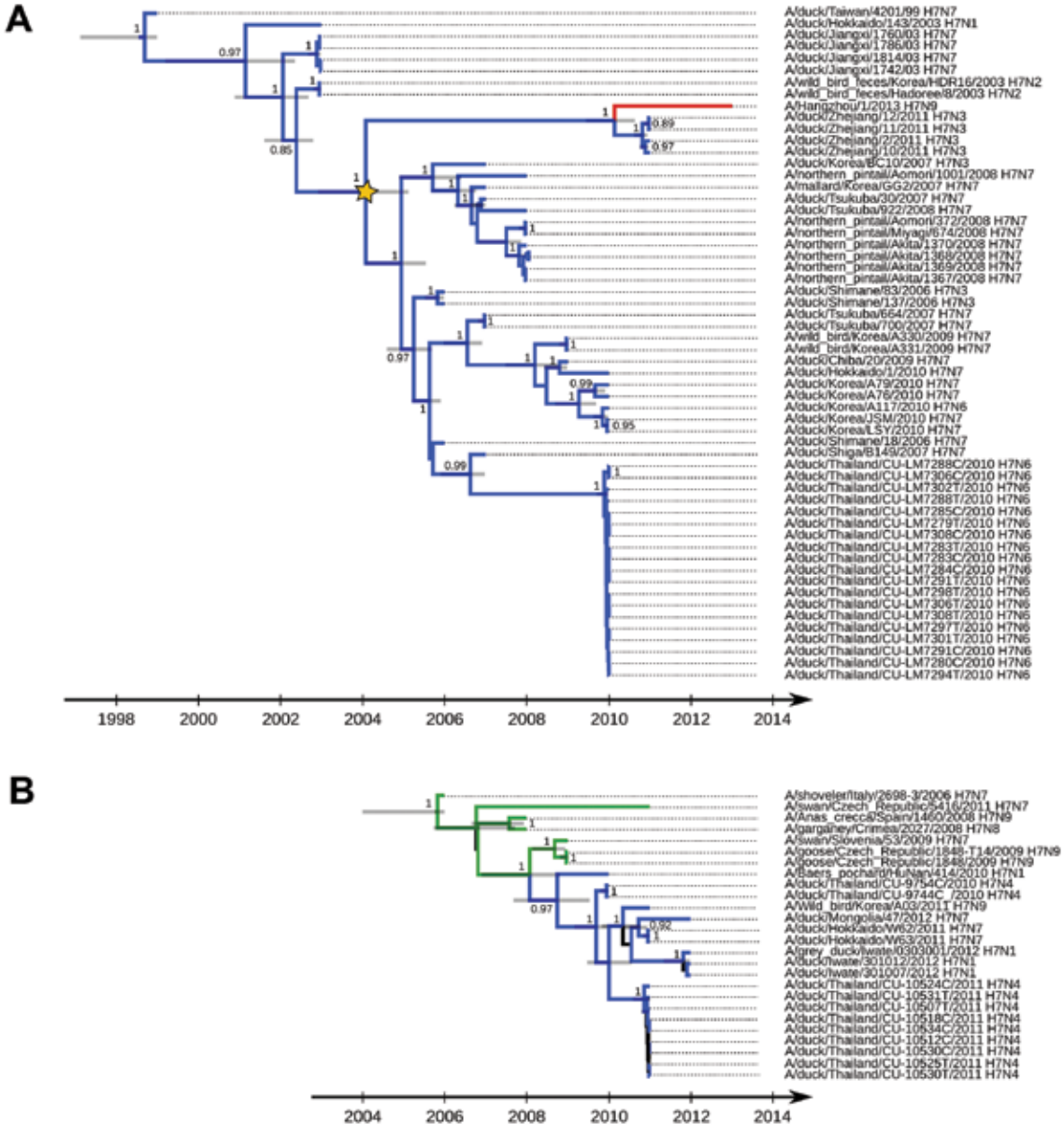

Figure 1. Maximum clade credibility trees for co-circulating influenza A virus H7 subtype genetic lineages, eastern Asia. A) Clade A. B) Clade B. Values along the branches are posterior probability values >0.8. Gray bars indicate 95% highest posterior density for times of the most recent common ancestors; blue indicates viruses isolated in Asia; green indicates viruses isolated in Europe (details on locations and associated posterior probabilities are shown in the online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0609-Techapp1.pdf); red indicates A/Hangzhou/1/2013(H7N9) virus; and yellow star indicates most recent common ancestral influenza virus among A/Hangzhou/1/2013(H7N9) virus, Zhejiang domestic ducks viruses (H7N3), and influenza A virus H7 subtype circulating in wild birds.

virus subtypes H5, H6, and H7. Consistent with results of other studies (*1,2,5,6*), we found that neuraminidase of A/Hangzhou/1/2013(H7N9) virus was closely related to that of A/wild bird/Korea/A3/2011(H7N9) virus (*9*). However, our estimate of the time of the most recent common ancestral influenza A virus between these 2 viruses was earlier (2008) than suggested (*6*).

Conclusions

Our findings suggest that neuraminidase of human influenza A(H7N9) virus might have originated from influenza A(H11N9) viruses that circulated in eastern China, although limited information about influenza A virus N9 subtypes circulating in wild birds in this region represents a major challenge to identifying the donor of influenza A(H7N9) virus neuraminidase. Reassortments between influenza A(H11N9) viruses from Asia and influenza A(H1N3 and H7N3) viruses circulating in live-poultry markets in Zhejiang Province were documented in domestic duck in 2011 in nearby Jiangsu Province (A/duck/Jiangsu/10-d4/2011(H11N3) (*10*) In a similar fashion, influenza A(H7N9) virus could have resulted from silent circulation and reassortment between influenza (H7N3 and H11N9) viruses in live-poultry markets in the Shanghai region.

As in a study on influenza A virus H7 subtype evolution in wild birds and poultry (*11*), we found no evidence of spillover of influenza A virus H7 subtype from domestic to wild birds and subsequent long-term maintenance in eastern Asia. Although we cannot formally exclude that local

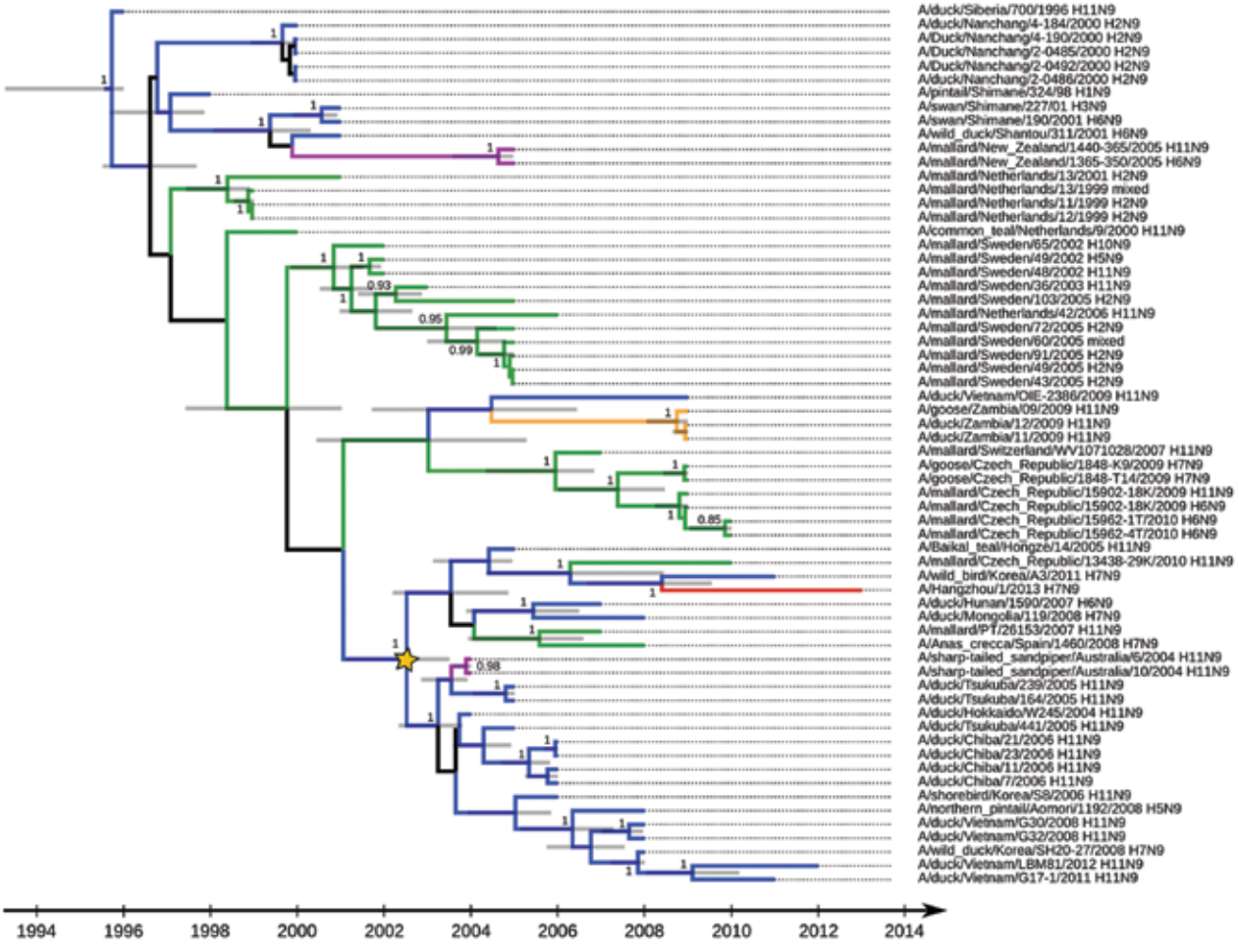

Figure 2. Maximum clade credibility tree for influenza A virus N9 subtype genetic lineages in Eurasia. Values along branches are posterior probability values >0.8. Gray bars indicate the 95% highest posterior density for times of the most recent common ancestors. Blue indicates viruses isolated in Asia; green indicates viruses isolated in Europe; purple indicates viruses isolated in Oceania; orange indicates viruses isolated in Africa (details on locations and associated posterior probabilities are shown in the online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0609-Techapp1.pdf); red indicates A/Hangzhou/1/2013(H7N9) virus; and yellow star indicates the basis of influenza A(H11N9) virus genetic lineage from Asia.

transmission of influenza A virus from domestic to wild birds has occurred, lack of evidence for reintroduction of poultry-adapted viruses into wild birds suggests there has been little to no dissemination of influenza A(H7N9) virus by waterfowl along their migratory flyways. Increasing adaptation of this virus to mammals (*2*) is unlikely to favor spillover and spread by migratory birds. However, development and maintenance of influenza A virus surveillance programs for wild waterfowls worldwide are needed to confirm this possibility (*12,13*).

In eastern Asia, 2 major influenza A virus H7 subtype genetic lineages have recently circulated in wild and domestic birds, and there has been potential replacement of the older lineage (clade A). Hemagglutinin of influenza A(H7N9) virus belongs to clade A. Genetic data indicate that the most recent ancestral wild bird–origin virus for A/Hangzhou/1/2013(H7N9) virus and Zhejiang domestic duck viruses can be dated to 2004. The influenza A(H11N9) virus that circulated in eastern Asia for ≈10 years, with associated intercontinental gene flows and reassortments, may be the donor of influenza A(H7N9) virus neuraminidase. Hosts and areas in which ancestral viruses have been maintained are unknown. However, influenza A(H7N9) virus probably emerged after a long silent circulation in live poultry markets in eastern Asia.

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Variant Human T-cell Lymphotropic Virus Type 1c and Adult T-cell Leukemia, Australia

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Human T-cell lymphotropic virus type 1 is endemic to central Australia among Indigenous Australians. However, virologic and clinical aspects of infection remain poorly understood. No attempt has been made to control transmission to indigenous children. We report 3 fatal cases of adult T-cell leukemia/lymphoma caused by human T-cell lymphotropic virus type 1 Australo-Melanesian subtype c.

The human T-cell lymphotropic virus type 1 (HTLV-1) currently infects at least 5–10 million persons worldwide; however, this oncogenic retrovirus is not ubiquitous, and areas of high endemicity are typically separated by areas where infection is uncommon (*1*). Although 4 major molecular subtypes in specific geographic areas have been described, epidemiologic and clinical associations have been best documented for the HTLV-1a subtype, which predominates in the Caribbean region and Japan. Among HTLV-1 carriers in these regions, adult T-cell leukemia/lymphoma (ATLL) will ultimately develop in 1%–5% (*2*). Few clinical details are available with regard to infection with the Australo-Melanesian HTLV-1c subtype, which is restricted to impoverished indigenous populations in Australia and the neighboring islands of Oceania.

In Australia, HTLV-1 carriers were first reported among indigenous residents of remote desert communities in 1988 (*3*). The sole published HTLV-1 nucleotide sequence from an indigenous Australian belongs to the Australo-Melanesian HTLV-1c subtype (*4*). Genetic characterization of the few available HTLV-1c subtype strains indicates that they are relatively divergent compared with the Cosmopolitan HTLV-1a prototype. Within the *env* gene and long terminal repeat regions, for example, 7%–10% divergence has been demonstrated at the nucleotide level (*5*,*6*). Background prevalence rates among indigenous central Australian adults are thought to be from 7.2% through 13.9% (*7*,*8*). However, among those admitted to the only regional hospital, the seropositivity rate approaches 40% (*8*), and rates are even higher among patients >45 years of age (49.3% for men; 38.5% for women) (*8*). The predominant mode of transmission among indigenous Australians is thought to be through breast-feeding (*8*).

In other populations, early acquisition of HTLV-1 infection is associated with an increased risk for ATLL (*2*). High HTLV-1 prevalence rates in some indigenous Australian and Melanesian communities coupled with frequent early childhood infection with HTLV-1 should therefore be associated with a correspondingly high risk for ATLL, yet few cases of HTLV-1–associated complications have been reported from Australasia (*9*,*10*). Indeed, the clinical significance of HTLV-1 infection in Australia has been questioned, and no attempt has been made to control virus transmission among the indigenous population (*8*). We report 3 cases of ATLL in indigenous Australian patients at the Alice Springs Hospital, central Australia, in 2002 and 2010. Case-patients 1 and 3 (Aus-NR and Aus-GJ) originated from the same community, ≈450 km from case-patient 2 (Aus-GM).

The Study

The first case-patient, a 68-year-old indigenous Australian man, reported a several-month history of limb pain, abdominal pain, and diarrhea. Peripheral blood examination revealed lymphocytosis (22.4×10^9/L) with atypical lymphoid cells. The patient was seropositive for HTLV-1 by Western blot and positive for hepatitis B surface antigen. Flow cytometry of cells obtained by bone marrow and lymph node biopsies revealed a population of CD2+, CD3+, CD4+, CD5–, CD7-, CD8-, CD25+ cells (Figure 1, Appendix, panels A, B, wwwnc.cdc.gov/EID/article/19/10/13-0105-F1.htm).

Histopathologic appearance was consistent with a diagnosis of ATLL, and T-cell receptor rearrangement studies revealed a monoclonal band. The case was complicated by a bloodstream infection with viridans group streptococci. This patient died of liver failure, which had complicated the sepsis, 22 days after admission.

The second case–patient, a 58-year-old indigenous Australian woman, reported a 1-week history of back pain and malaise. Peripheral blood examination revealed hyperlymphocytosis (58×10^9/L) caused by CD2+, CD3-, CD4+, CD5+, CD7-, and CD8- atypical lymphoid cells (Figure 1,

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Appendix, panel C). The patient was seropositive for HTLV-1 by Western blot. Corrected serum calcium was 3.5 mmol/L. Plain radiographs revealed lytic bone lesions, which were also apparent on computed tomography imaging of her abdomen, as was extensive lymphadenopathy and a para-aortic mass (Figure 1, Appendix, panels D, E). The results of bone marrow biopsy were consistent with ATLL, and T-cell receptor rearrangement studies revealed a monoclonal band. This patient died of sepsis 13 days after admission.

The third case–patient, a 74-year-old indigenous Australian man, reported a history of back pain; laboratory investigations revealed hypercalcemia, acute renal failure, and pancytopenia. A peripheral blood film revealed small lymphocytes with cloverleaf-shaped nuclei and prominent nucleoli. The patient was HTLV-1 seropositive by Western blot. Bone marrow biopsy revealed that the marrow was almost totally replaced by a population of CD25+ lymphoid blast cells, consistent with a diagnosis of acute ATLL. A monoclonal T-cell receptor band was observed from fresh tissue. The case was complicated by *Staphylococcus aureus* bacteremia, and the patient died of overwhelming sepsis 41 days after diagnosis.

Molecular characterization of the viral strain was possible for patients 1 and 2. The entire HTLV-1 *env* gene was obtained by using DNA extracted from peripheral blood buffy coats, providing a complete *env* gene sequence of the c subtype characterized from indigenous Australians. Phylogenetic analysis was performed by using a 1,386-bp fragment of *env* gene sequences (from patients Aus-NR and Aus-GM) and 28 primate T-lymphotropic virus type 1 sequences. These sequences cluster with the Australo-Melanesian c-subtype (Figure 2) and are closely related to the sole, but incomplete, HTLV-1 strain (MSHR-1) from the previously described case of an indigenous Australian (*4*). This phylogenetic study also demonstrates the existence of at least 2 subgroups strongly supported phylogenetically within the Melanesian clade: 1 comprising strains from Australia and Papua New Guinea and 1 comprising strains from Vanuatu and the Solomon Islands (Figure 2).

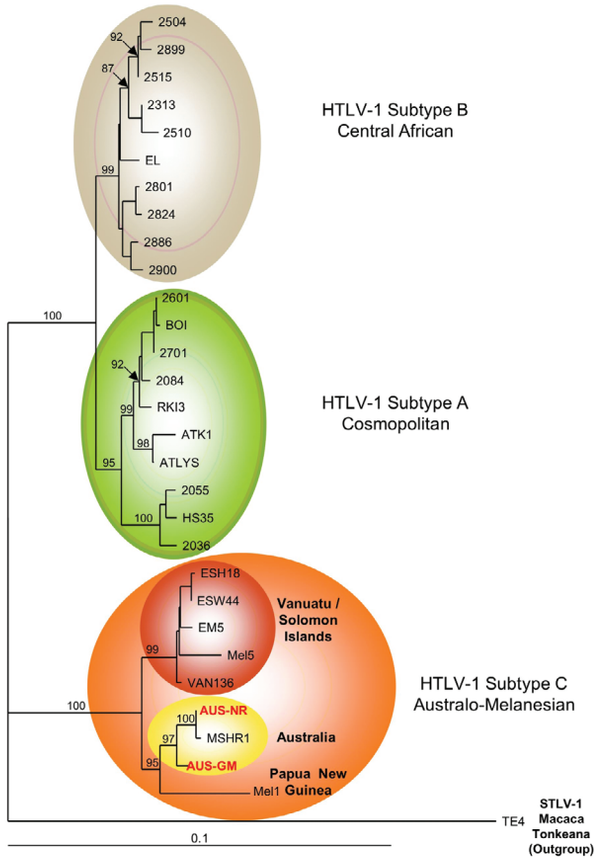

Figure 2. Phylogenetic tree generated on a 1,386-bp fragment of the env gene for 30 primate T-lymphotropic virus type 1 available sequences, including the 2 sequences generated in this work (in red boldface). The lymphotropic virus type 1 strains were aligned with the DAMBE program (version 4.2.13). The final alignment was submitted to the ModelTest program (version 3.6) to select, according to the Akaike Information Criterion, the best model to apply to phylogenetic analyses. The selected model was the Tamura Nei. Bootstrap values were calculated for 1,000 replicates, and the numbers at some nodes of the tree (bootstrap values) indicate frequencies of occurrence for 100 trees. The TE4 strain from Macaca tonkeana macaques was used as an outgroup. The branch lengths are drawn to scale, and the scale bar indicates nucleotide replacements per site. Three of the 4 major HTLV-1 subtypes are shown. Subtype c corresponds to Australo-Melanesian HTLV-1c and includes the 2 env sequences (Aus-NR and Aus-GM; GenBank accession nos. KF242508 and KF242509, respectively), which were obtained from patients 1 and 2, respectively. HTLV, human T-lymphotropic virus; STLV, simian T-lymphotropic virus.

Conclusions

Elsewhere, HTLV-1–associated ATLL directly causes death in as many as 1 in 20 HTLV-1 carriers (*2*). Each patient from central Australia reported here was seropositive for HTLV-1 according to stringent Western blot criteria, and the HTLV-1c variant strain was identified for both patients from which mononuclear cell DNA was available. All 3 patients died within 6 weeks of diagnosis. The patients described here had been admitted to the only regional hospital; others might have died in remote areas before transfer. Nevertheless, the estimated crude mean annual incidence of ATLL among ≈1,400 adult HTLV-1 carriers in central Australia (23.6 cases/100,000 population) is similar to that of the Caribbean region (20 cases/100,000 population) (*11*). Rates are somewhat higher in Japan (86 cases/100,000 population) (*11*); however, many indigenous Australians die during the latent period required for malignant transformation (*8*), and ATLL incidence rates in central Australia might rise as life expectancy for indigenous Australians improves.

The paucity of clinical information regarding HTLV-1–associated diseases among indigenous Australasians might reflect these persons' limited access to medical care rather than intrinsic differences in the oncogenic potential of the HTLV-1c Australo-Melanesian subtype. Indeed, social deprivation can increase the risk for adverse outcomes among HTLV-1 carriers (*8*). For example, environmental contamination resulting from poor sanitation increases risk for co-infection with *Strongyloides stercoralis* roundworms, which shortens the latent period for malignant transformation (*12*). Whether co-infection with *S. stercoralis* parasites contributed to the development of ATLL in the patients reported here is uncertain. However, *S. stercoralis* parasites are endemic to central Australia (*8*), and anthelminthic prophylaxis has been suggested as a way to reduce risk for ATLL among HTLV-1 carriers residing in other *S. stercoralis* parasite–endemic areas (*13*). Social deprivation might also increase the risk for HTLV-1–associated pulmonary disease. In residents of developed countries, this disease is typically subclinical; however, in indigenous Australians, it appears as multifocal bronchiectasis, resulting in substantial illness and death at a median of 44.5 years of age (*14*).

The occurrence of 3 cases of ATLL in indigenous central Australian adults, together with previous studies that have demonstrated high HTLV-1 seropositivity rates (*8*), endemic strongyloidiasis (*8*), and high prevalence of HTLV-1–associated bronchiectasis (*14*), confirm that HTLV-1–associated diseases contribute to illness and death among indigenous Australians. These findings demand a public health response to control HTLV-1 transmission, particularly to indigenous children who are at greatest risk.

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Transition in the Cause of Fever from Malaria to Dengue, Northwestern Ecuador, 1990–2011

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In tropical areas, the predominant cause of fever has historically been malaria. However, by 2011, dengue was identified in 42% of febrile patients in northwestern Ecuador and malaria in none. This finding suggests a transition in the cause of fever from malaria to other illnesses, such as dengue.

Clinical and public health decisions about infectious diseases depend on the specific agents of infection. The predominant cause of fever in tropical areas has historically been malaria. However, dengue is becoming more of a concern as its geographic range expands, despite efforts to control the spread of the main vector, *Aedes aegypti* mosquitos (*1*). In the past 2 decades, dengue has expanded from urban areas, the focal point of endemic and epidemic activity, to more rural regions (*2*). At about the same time, malaria incidence has decreased by 17% globally; in Ecuador, it decreased by >75% (*3*). Etiologic transitions like these require timely changes in treatment and intervention strategies.

In Ecuador, reported dengue cases reemerged in 1988, peaked in 2000, and exhibited typical oscillatory patterns during the next 12 years (Figure 1). During the 1990s, dengue spread to the province of Esmeraldas; after 1998, when *Ae. aegypti* mosquitoes became established, dengue spread into the rural Esmeraldan community of Borbón (*4*). According to official data, the number of confirmed dengue cases in Borbón remained low until 2009 (*5*). Although reporting bias probably affects these data, the reported pattern is consistent with the national epidemic profile for 2009–2010 (Figure 1). During this same period, malaria prevalence decreased steadily in Esmeraldas and throughout Ecuador (Figure 2).

In Ecuador, since the 1988 reintroduction of dengue virus, all 4 serotypes have circulated. In contrast, dengue virus type 1 (DENV-1) was introduced into Esmeraldas in 1990 and was the only documented serotype circulating until 2004, when DENV-4 was identified. DENV-4 was thought to arrive in Esmeraldas from Colombia and subsequently spread into the rest of Ecuador (*6*). The most likely reason Esmeraldas was a point of DENV introduction is the increased building of roads during 1996–2002; these roads connected Esmeraldas to Colombia and the rest of Ecuador and are a major reason for pathogen transmission (*7*,*8*).

Most surveillance data are predicated on clinical manifestations. Like many other infectious diseases, dengue infection follows an unpredictable clinical evolution, ranging from inapparent or mild fever to severe or fatal hemorrhagic disease (*9*). Patients with acute dengue infection experience broad clinical signs and symptoms (e.g., fever, headache, myalgia, and abdominal pain), often characterized as an acute undifferentiated febrile illness, which can be caused by a wide array of pathogens, including *Leptospira* spp., *Plasmodium* spp., *Rickettsia* spp., and DENV (*10*); therefore, without laboratory tests, dengue infections are often misdiagnosed (*11*).

Given the recent downward trend in the number of malaria cases and the limited official data available on confirmed dengue cases in rural Esmeraldas, our objective was to determine whether dengue infection is present in febrile patients at either the laboratory of the Servicio Nacional de Erradicación de Vectores Artrópodos (SNEM), where patients who suspect that they have malaria often go, or the Hospital Civil de Borbón (HCB), the main hospital in the region. We provide data from Borbón, a rural community in northwestern coastal Ecuador, as a case study of this etiologic transition of fever from malaria to other illnesses, such as dengue. In addition, we provide serotype-specific data that describe the directional flow of dengue at the regional and national levels.

The Study

At HCB, as part of the routine workup for febrile patients, serum samples were collected, stored at -20°C, and transported in liquid nitrogen to Quito for PCR. Hospital personnel also tested these samples by using a dengue IgM/IgG-capture ELISA with high specificity and sensitivity (Panbio, Waltham, MA, USA). At the SNEM laboratory, 2–4 drops of whole blood were collected on filter paper (Whatman 903, Kent, UK) from persons spontaneously seeking or referred for malaria diagnosis. Filter papers

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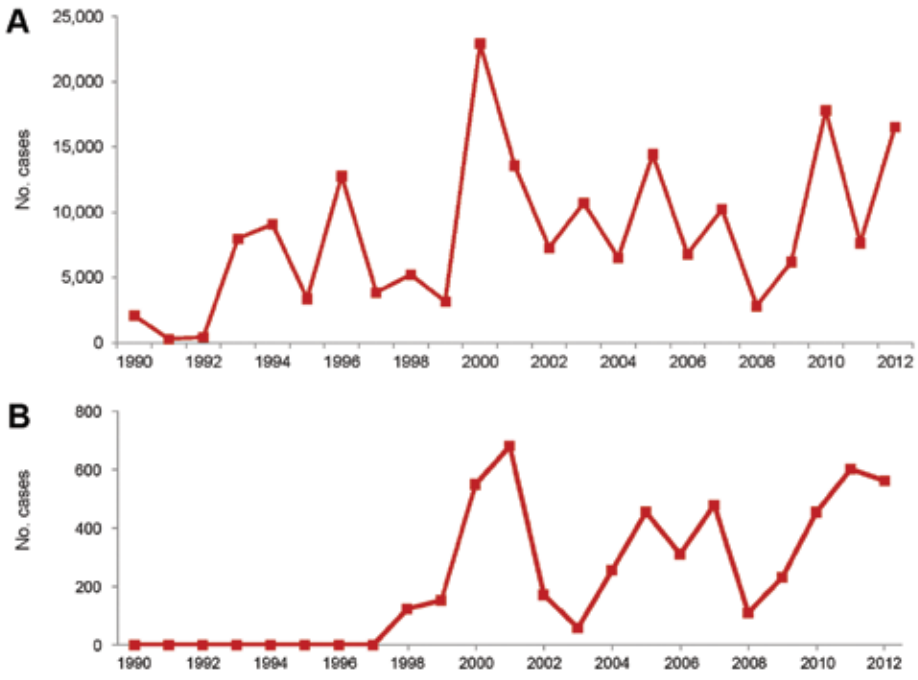

Figure 1. Suspected cases of dengue in Ecuador (A) and Esmeraldas Province (B), 1990–2012. Data from Annual Epidemiology Reports, Ministerio de Salud Pública del Ecuador.

were left to dry overnight, stored at 0°C in a zipper bag, and transported to Quito for analysis. DENV was amplified from total RNA from blood spots and serum as described (*12*,*13*). Enriched media from DENV 1–4 cultures were used as positive controls. Quality of RNA extracts was tested by using β-actin gene amplification. PCR products were sized in 1.5% agarose gel electrophoresis and SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) (1:10,000) under UV light. PCR product was sent to Functional Biosciences (Madison, WI, USA) for sequencing. All participants provided oral consent. The study was approved by institutional review board committees at the Universidad San Francisco de Quito (Quito, Ecuador) and the University of Michigan (Ann Arbor, MI, USA).

From July 2010 through February 2011, a total of 77 samples (36 serum, 41 blood spot) were collected from febrile patients. Of these 36 hospital patients, 10 were from Borbón and 26 were from the surrounding communities; patients were 2–74 years of age (median 19 years), and fever duration was 3–20 days (median 6 days). Six (17%) of the 36 serum samples and 7 (17%) of the 41 blood spots were positive for dengue by PCR; whereas 10 (29%) of the 34 serum samples tested by ELISA were positive for dengue IgM (Table). The ages of the 10 patients with positive

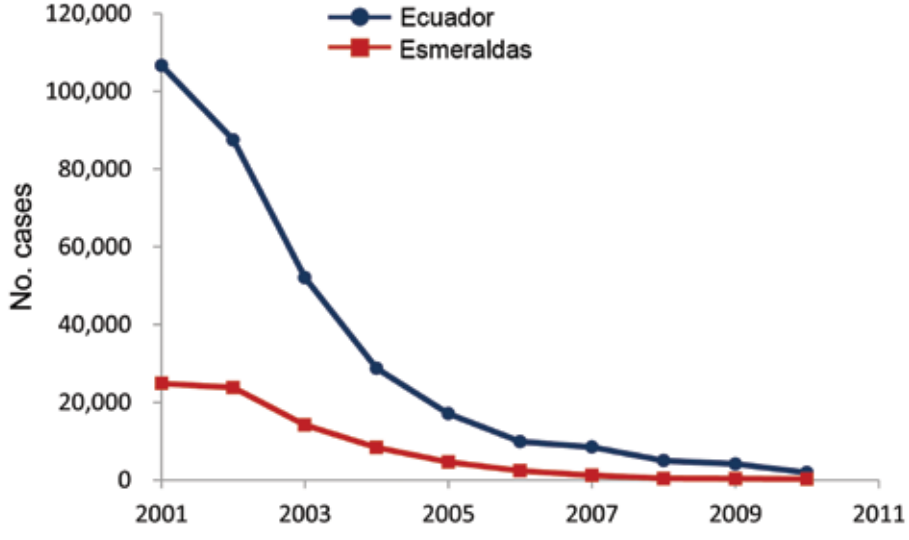

Figure 2. Confirmed malaria cases in all of Ecuador and in the Esmeraldas Province, 2000–2010. Data from the Department of Epidemiology, Servicio Nacional de Erradicaciòn de la Malaria, Ministerio de Salud Pública del Ecuador.

Table. Dengue virus in serum samples and blood spot samples, Ecuador*

Specimen	No. specimens	No. positive		No. each dengue virus subtype			
		ELISA	RT-PCR	1	2	3	4
Serum	36	10†	6‡	0	5	1	0
Blood spot	41	Not applicable	7§	0	3	5	0

*RT-PCR, reverse transcription PCR
†Only 34 serum samples were tested by ELISA; only 1 of the 10 positive samples was also positive by PCR.
‡Five samples were from male patients.
§Three samples were from male patients. One patient was co-infected with both serotypes.

results by ELISA were 3–63 years (median 38 years), and fever duration was 4–13 days (median 6.5 days). Although positive PCRs are sufficient indicators of acute infection, the positive IgM reflects a cumulative incidence over 60–90 days. Therefore, the 17% PCR-positive estimate is a lower bound, and the 42% estimate by PCR or ELISA is an upper bound. The DENVs in the 13 samples that were positive by PCR were either serotype 2 or 3; samples from 1 patient contained both serotypes, probably resulting from a co-infection. Each institution (HCB and SNEM) submitted samples from the same 2 patients, but only 1 was positive for dengue by PCR. During this same period, DENV-1, -2, and -4 were detected in coastal cities in other regions of Ecuador (DENV-3 has not been reported in Ecuador since 2009 [*14*]). These data suggest that DENV-3 was introduced into the study region from another country. Colombia is a likely source, given the proximity of the study region to Colombia and the fact that DENV-3 was circulating in Colombia; but without sequence data, the geographic location of the source cannot be confirmed.

Conclusion

This study provides evidence of a transition of febrile disease etiology from *Plasmodium* spp., traditionally highly prevalent in this area, to reemerging pathogens, such as DENV. Other possible fever-causing agents for which we did not test include *Leptospira* spp., *Rickettsia* spp., *Brucella* spp., and viral agents of encephalitis (*11*,*15*). Given that malaria has historically been the predominant cause of fever in the developing world, febrile patients are often triaged toward malaria treatment, especially in resource-poor areas. Moreover, the awareness of malaria is embedded culturally and behaviorally in these communities: having a fever is equated with having malaria. Of the 40 febrile patients who sought treatment for malaria, none had malaria but 17% had dengue (positive for DENV by PCR). Further research is needed to refine this estimate and explore how the Ministry of Health and the general population should be directed to manage cases of fever during this etiologic transition. The need to bring attention to this transition is exemplified by our finding of DENV-3 in our study site; this serotype has not been isolated in other parts of Ecuador since 2009. Esmeraldas, therefore, may be a major source of newly introduced dengue serotypes into Ecuador.

Acknowledgments

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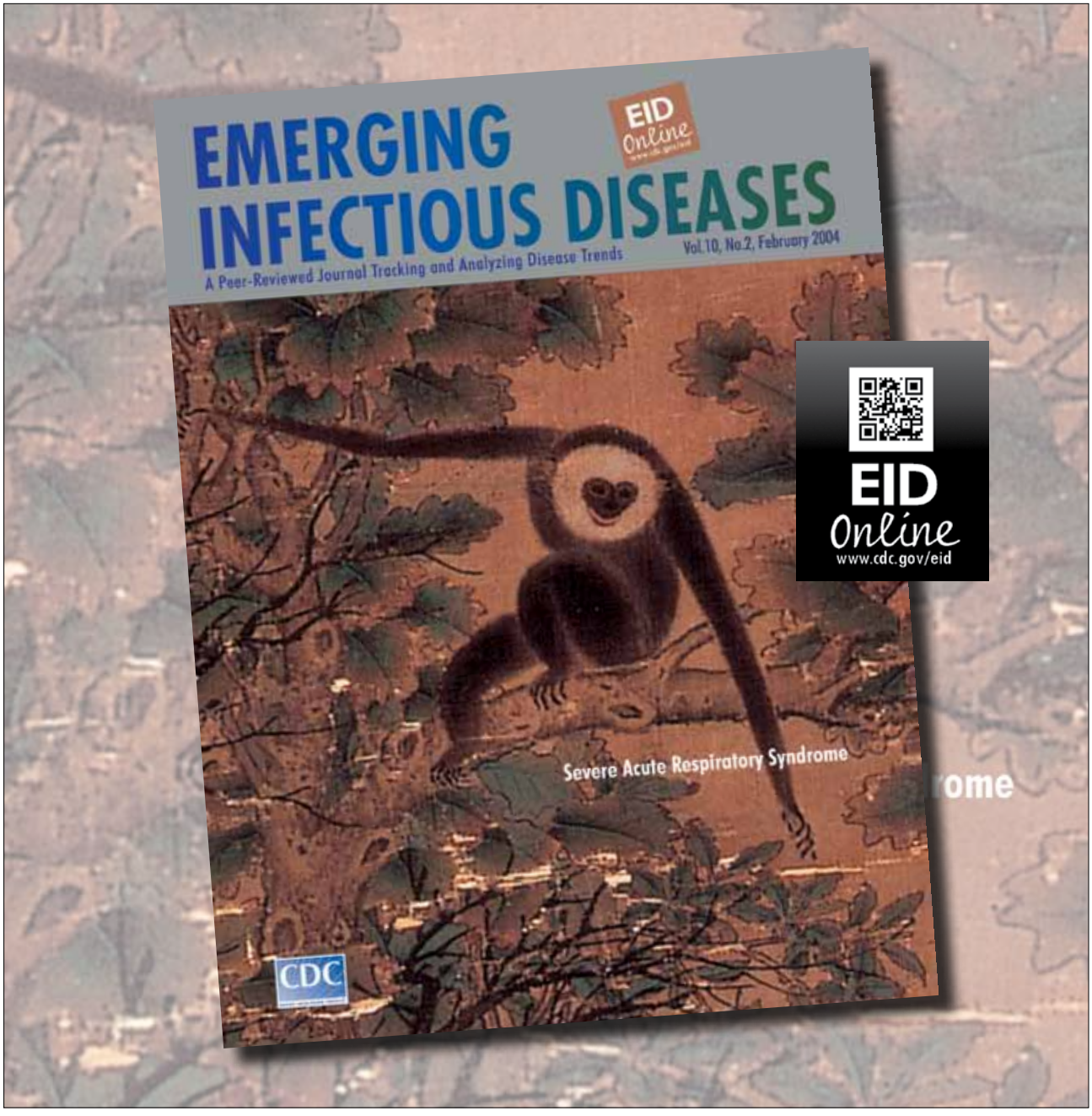

Use of Primary Care Data for Detecting Impetigo Trends, United Kingdom, 1995–2010

Laura J. Shallcross, Irene Petersen, Joe Rosenthal, Anne M. Johnson, Nick Freemantle, and Andrew C. Hayward

Using a primary care database, we identified a major increase in impetigo in the United Kingdom during 1995–2010. Despite a doubled rate of primary care consultations, this increase was not identified by routine surveillance. Primary care databases are a valuable and underused source of surveillance data on infectious diseases.

Impetigo is a common, superficial bacterial infection primarily caused by *Staphylococcus aureus*. It is the fourth most common dermatologic condition among children seen in general practice; although most infections are mild, outbreaks have a considerable negative effect because infected children may be barred from attending schools and nurseries (*1*).

In the United Kingdom, hospitalizations for impetigo increased 5-fold from 1989–1990 through 2003–2004; among children, the increase was 12-fold (*2*). To investigate whether this increase was fueled by increasing rates of infection in the community rather than increased pathogenicity, we used a large and nationally representative primary care database to calculate time trends in incidence of consultation and medications prescribed for impetigo.

The Study

We examined electronic patient records from The Health Improvement Network (THIN) database, a source of detailed clinical information about patient primary care consultations (*3*). In the United Kingdom, 98% of the population is registered with a general practitioner (a primary care physician who provides advice, treatment, and prescriptions and acts as a gatekeeper to specialist services) (*4*). Participating practices enter demographic and clinical data into the practice database by using Vision software (www.inps4.co.uk) every time a consultation takes place, generating a longitudinal medical record. Since 1990, symptoms, diagnoses, treatments, and referrals have been recorded by use of a hierarchical system of >103,000 Read codes (*5*). Prescriptions are recorded by using Multilex (www.fdbhealth.co.uk/solutions/multilex) drug codes, which link each drug formulation to the British National Formulary, a compendium of drugs arranged by system into 15 chapters. THIN contains the medical records of >3.7 million current patients (*3*) and is broadly representative of the UK population (*6*). Prescription and consultation rates in the dataset are comparable to those recorded by national statistics and external data sources (*7*,*8*). Adequacy of death data recording is assessed by identifying the date at which mortality rates recorded by the practice correspond to the national age and sex standardized mortality rate, defined as the acceptable mortality recording date. We included data from patients from practices that met acceptable mortality recording criteria and were fully computerized (*9*). Persons were eligible for study inclusion if they were registered with a participating practice from January 1, 1995, through December 31, 2010.

Read code lists were developed to identify patients seeking care for impetigo. A drug code list was developed to identify patients for whom topical fusidic acid (with or without a topical steroid) was prescribed, based on the relevant chapter of the British National Formulary.

To assess time trends, we included the first consultation for impetigo for patients 0–14 years of age or the first prescription of fusidic acid; that is, patients were counted once per practice. Patients left the study on the earliest of the following dates: date of consultation or prescription, date of leaving the practice, date of death, or date the study ended (December 31, 2010). We used Poisson regression to calculate the incidence of first consultations or prescriptions per year. The denominator was the total number of person-years contributed by patients in the sample population for each corresponding calendar year.

The THIN program of anonymized data provision for researchers was approved by the National Health Service South East Multi-Centre Research Ethics Committee in 2002. This study was approved by the THIN scientific review committee, reference 11–504.

During 1995–2010, a total of 130,095 children 0–14 years of age were seen by a general practitioner for impetigo. The annual incidence of infection increased from 1,646 (95% CI 1,561–1,733) consultations per 100,000 person-years in 1995 to 3,106 (95% CI 3,048–3,165) per 100,000 person-years in 2001 (Figure 1) and declined thereafter to 1,447 (95% CI 1,413–1,481) per 100,000 person-years in 2010. The incidence of fusidic acid prescription for the total population increased from 1,287 (95% CI 1,256–1,318) prescriptions per 100,000 person-years in 1995 to 2,308 (95% CI 2,289–2,326) per 100,000 person-years in 2003

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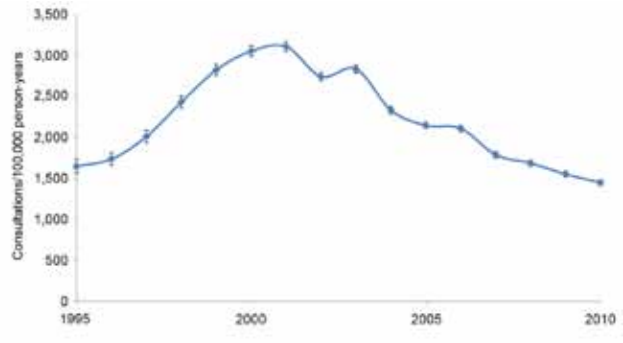

Figure 1. Rates of general practitioner consultation for impetigo among children 0–14 years of age, United Kingdom, 1995–2010. Error bars indicate 95% CIs.

and stabilized thereafter (Figure 2). Prescriptions were most frequently issued for children 0–14 years of age; incidence peaked in 2003 at 4,911 (95% CI 4,843–4,980) prescriptions per 100,000 person-years. At the peak of the epidemic, there were ≈130,000 more general practitioner consultations for impetigo; this estimate is based on an estimated population of 9,375,100 children <15 years of age in England in 1995 and 9,282,700 in 2001 (*10*).

Conclusions

A major increase in impetigo in children in the United Kingdom was not detected by routine surveillance data. The increased number of infections placed a substantial burden on primary care, adding 130,000 consultations in England at the peak of transmission. In England and Wales, the Royal College of General Practitioners Research and Surveillance Centre network of ≈100 sentinel practices is responsible for alerting general practitioners to major trends in the incidence of common conditions, such as influenza and impetigo, by providing weekly reports of disease incidence (*11*). Although this system detected a comparable increase in the rate of consultations for impetigo, the data were not used to alert general practitioners about a major increase in impetigo in the community because the system is designed to detect rapid changes in incidence, such as occur during an influenza epidemic.

Over the past decade, several European countries have reported a rise in general practice consultations for impetigo (*12*,*13*). Many European countries have established primary care databases, such as the Information System for the Development of Research in Primary Care in Catalonia (*14*) or the Health Search Database in Italy (*15*). Although these databases are smaller than the THIN database (*3*), they are sufficiently large and well-established to be used to examine national consultation and prescription trends in infectious diseases, offering the potential for a powerful international infectious disease surveillance network.

This study's strengths lie in its scale and the fact that the database is nationally representative, containing the medical records of ≈6% of the UK population (*4*). The study's limitations lie in the fact that the database was designed for patient management, not research. We acknowledge that comparing population rates for consultation and prescriptions might not accurately represent the association between prescriptions and disease at an individual level. Patients seeking consultation for impetigo were identified by diagnostic Read codes, and some general practitioners might prescribe fusidic acid without recording a diagnostic code. Identifying patients by Read code alone might underestimate incidence, whereas identifying patients by prescription data might lead to an overestimation because drugs are not always prescribed for a single condition. The actual incidence of impetigo in the community might have been higher because not all persons with impetigo would have consulted a general practitioner.

Although changes in health-seeking behavior or data recording could underlie the increased consultation rate, the fact that similar increases were reported from other primary and secondary care datasets suggests that our findings are not artifacts. The Read codes used to identify patients were unchanged throughout the study period, and we are unaware of any changes in clinical practice that would lead to an increased tendency to diagnose impetigo.

Impetigo is frequently dismissed as a mild infection that spontaneously resolves with a good outcome (*1*). By contrast, this study suggests that an undetected increase in impetigo in the community drove a major increase in hospital admissions of children in England from 1989–1990 through 2003–2004. Awareness of this epidemic by general practitioners could have triggered development of specific guidelines on the management of this condition, potentially improving treatment outcomes and reducing hospital admissions. Routinely collected primary care data are an underused and potentially rich source of information about infectious diseases in the community. We should do more to find novel ways of

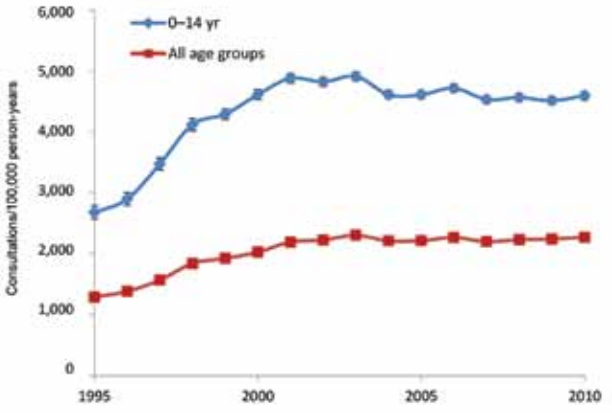

Figure 2. Rates of fusidic acid prescription by general practitioners, United Kingdom, 1995–2010. Error bars indicate 95% CIs.

incorporating this information into international surveillance networks and using it to guide evidence-based treatment and prescribing decisions in primary care.

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Primary Multidrug-Resistant *Mycobacterium tuberculosis* in 2 Regions, Eastern Siberia, Russian Federation

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Of 235 *Mycobacterium tuberculosis* isolates from patients who had not received tuberculosis treatment in the Irkutsk oblast and the Sakha Republic (Yakutia), eastern Siberia, 61 (26%) were multidrug resistant. A novel strain, S 256, clustered among these isolates and carried *eis*-related kanamycin resistance, indicating a need for locally informed diagnosis and treatment strategies.

In 2010, tuberculosis (TB) prevalence in the Russian Federation was 136 cases per 100,000 population; the estimated proportion of multidrug resistance, defined as resistance to isoniazid and rifampin in the absence of prior treatment (primary MDR TB), was 18% (*1*). However, at the subnational level, primary MDR TB might be highly variable; in oblasts or republics with continuous surveillance data, drug resistance varies from 5.4% to 28.3% (*2*). These data are predominantly from the western half of the country and do not include eastern Siberia.

In 2009, in the Irkutsk oblast in eastern Siberia, TB prevalence was 373 cases per 100,000 population and HIV prevalence was among the highest in the Russian Federation (*3*,*4*). In contrast, in the sparsely populated neighboring Sakha Republic (Yakutia), TB prevalence was lower (188 cases/100,000 population) and HIV was thought to be scarce (*4*). Molecular typing has found that more than half of the *Mycobacterium tuberculosis* isolates from the Russian Federation are the Beijing genotype, a pandemic lineage associated with MDR phenotype and characteristic drug-resistance mutations; prevalence of this genotype in Irkutsk is high (*5*,*6*). However, such investigation has not been performed in Yakutia. Given the distinct sociocultural patterns between Irkutsk and Yakutia, we hypothesized that the molecular epidemiology and drug-resistance patterns of *M. tuberculosis* from patients with primary MDR TB would be regionally distinct.

The Study

From November 2008 through May 2010, *M. tuberculosis* isolates were cultured during routine care of adults ≥18 years of age with primary TB and no history of treatment. The patients were from 2 regional referral centers, the Irkutsk Regional TB-Prevention Dispensary and the Research Practice Center for Phthisiatry (Yakutia); the study was approved by the institutional review boards at the University of Virginia and Irkutsk State Medical University.

Initial pretreatment isolates were grown on Lowenstein-Jensen agar slants and identified to species in accordance with World Health Organization recommendations. Drug susceptibility was tested by absolute concentration method on agar slants; drugs tested were rifampin (critical concentration 40 μg/mL), isoniazid (1 μg/mL and 10 μg/mL), ethambutol (2 μg/mL), streptomycin (10 μg/mL), ethionamide (30 μg/mL), and kanamycin (30 μg/mL). Susceptibility to a fluoroquinolone and pyrazinamide was not routinely tested. DNA extraction was performed on all isolates, followed by 12-loci mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) analysis (*7*) and further lineage definition by region of difference deletions, or for Ural strains as described (*5*). Phylogenetic tree construction was based on the MIRUVNTR*plus* database (*8*), and VNTR international type numbers were confirmed on the SITVIT database (*9*). DNA from MDR isolates was amplified and sequenced for the known drug-resistance determining regions *katG*, *inhA*, *rpoB*, *embB*, *gyrA*, *rrs*, and *eis* by using methods described by the Centers for Disease Control and Prevention (*10*). For *pncA*, the entire open reading frame and upstream promoter region were amplified. Sequences were compared with published sequences for *M. tuberculosis* H37Rv by using GeneDoc version 2.7.0.

Among 235 patients with primary TB (130 from Yakutia, 105 from Irkutsk), isoniazid monoresistance was found in isolates from 16 (12%) from Yakutia and 19 (18%) from

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Irkutsk (p = 0.27). Multidrug resistance was found for 61 patients (36 [28%] from Yakutia and 25 [24%] from Irkutsk) (p = 0.55). Mean age (± SD) for these 61 patients was 33 (± 12) years, 40 (66%) were male, and these characteristics did not differ significantly between patients from Irkutsk and from Yakutia. However, no HIV-infected patients were identified from Yakutia compared with 11 (44% with MDR TB) from Irkutsk (p<0.001). Twelve MDR TB patients from Irktutsk died (outcome unknown for the other 13 patients), including all with HIV, compared with 4 (11%) from Yakutia who died (p = 0.002). Follow-up varied and was limited mostly to inpatients.

Among all 235 patients with primary TB, strains of the Beijing family were significantly more common among those from Irkutsk (70 [67%]) than from Yakutia (40 [31%]) (p<0.001). However, strains found in Yakutia (S 256 [11%], T 8 [7%], and Ural 171 [5%]) were not found in Irkutsk (Table 1). The cluster of S 256 (MIRU profile 233325153325) was the most common among primary MDR TB isolates from Yakutia and was fully 86% MDR (Table 1; online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-1108-Techapp1.pdf).

Among isolates from patients with primary MDR TB, 51 (84%) were available for DNA sequencing: 27 from Yakutia and 24 from Irkutsk (Table 2; an expanded version of this table is available at wwwnc.cdc.gov/EID/article/19/10-1108-T2.htm). Among isoniazid-resistant isolates, the mutation in codon 315 of *katG* was present in 91%. Among rifampin-resistant isolates, mutations in the resistance-determining region of *rpoB* (codons 511–533) were present in only 79%. The *pncA* mutation was common across genotypes from both sites, occurring in 62% of isolates amplified. Notably, both isolates with mutation in *eis* from Yakutia occurred in MDR strains with the S 256 genotype and without *rrs* mutation.

Conclusions

In eastern Siberia, >25% of primary TB was MDR, equivalent to the highest proportion reported from the Russian Federation (*2*). However, regionally specific genotypic patterns and resistance mutations were identified. As expected, in Irkutsk primary MDR TB was driven by strains of Beijing lineage (*5*,*6*). Yet in the more geographically isolated population of Yakutia, a strain previously unidentified in the Russian Federation, S 256, had a MIRU profile recently found among Canadian Aboriginal populations (*11*). In Yakutia, S 256 was highly drug resistant and was the most common genotype among patients with primary MDR TB.

Although *rpoB* mutations were found in only 79% of rifampin-resistant isolates, these findings are consistent with those in a recent report from Novosibirsk oblast, which similarly included non-Beijing and S-family strains and found a sensitivity of only 63% for the *rpoB* mutation (*12*). Lack of phenotypic correlation can result from alternate mechanisms of resistance or imperfect conventional susceptibilities in Lowenstein-Jensen medium or from use of old drug stock. Such discrepancy necessitates urgent clarification because substitution of conventional susceptibility testing with molecular probe–based methods such as GeneXpert MTB/RIF (Cepheid, CA, USA) has been strongly advocated but would lead to dramatically different results and treatment regimens (*13*). Of note, isolates of the S 256 strain accounted for a proportion of the cases in which mutation in the promoter region of *eis* was associated with kanamycin resistance, but *rrs* was wild type. Commercial assays have focused on the *rrs* locus, which has greater sensitivity for amikacin, as the sole target for the class of injectable agents (*14*), yet in Eastern Siberia, the injectable agent available is kanamycin. Furthermore, we found a range of reported and unreported mutations across the entire *pncA* gene; most were point mutations resulting in amino acid substitution, but some strains had mutations that resulted in deletion or frameshift. Phenotypic methods and assays of functional pyrazinamidase activity should be performed in this region because results might have major implications for novel MDR TB drugs that work best with pyrazinamide (*15*).

Study limitations include selection bias of isolates from passive surveillance. We were unable to obtain detailed clinical information about all patients with primary TB, thus preventing adequate comparison of nongenotypic risk factors for MDR TB or establishment of definitive

Table 1. *Mycobacterium tuberculosis* genotype, by region, eastern Siberia, Russian Federation*

		Irkutsk			Yakutia		
MIRU-VNTR 12	Family/ MIT	No. (%) total, n = 105	No. (%) MDR, n = 25	p value	No. (%) total, n = 130	No. (%) MDR, n = 36	p value
223325153533	Beijing 16	32 (31)	7 (28)	<0.001	12 (9)	1 (3)	0.006
223325173533	Beijing 17	13 (12)	6 (24)	0.27	10 (8)	7 (19)	0.76
233325153325	S 256	0	0	<0.001	14 (11)	12 (33)	0.001
223125153324	T 8	0	0	NA	9 (7)	0	0.005
227225113223	Ural 171	0	0	NA	6 (5)	0	0.03
223325153433	Beijing 592	1 (1)	0	NA	4 (3)	0	0.38

*MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat (original 12-loci profile). Included genotypes found in ≥5 isolates only; MIT, MIRU–VNTR international type; MDR, multidrug-resistant tuberculosis (conventional resistance to isoniazid and rifampin); NA, not applicable. Significance determined by χ^2 analysis with Yates correction or Fisher exact test when appropriate.

epidemiologic links among clustered isolates. Furthermore, lack of conventional fluoroquinolone or pyrazinamide susceptibility testing limited comparison with *gyrA* and *pncA* mutations, respectively. Despite these limitations, this work characterizes severe isoniazid monoresistant and MDR TB in eastern Siberia among patients with no history of TB treatment. The regionally distinct phylogenetic patterns and certain drug-resistance mutations necessitate careful application of novel diagnostics and empiric therapeutic strategies.

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Table 2. Resistance mutations in *Mycobacterium tuberculosis* from 51 patients from Irkutsk and Yakutia, Russian Federation*

Drug, locus	Mutation, no. (% total)		Drug resistance, no. (% with mutation)
	Amino acid change	Nucleotide change	
Rifampin, *rpoB*	Ser531Leu 19 (37)		15 (79)
	Ser531Leu/Thr481Ala 1 (2)		0
	Ser 531Leu/Thr480Ile 2 (4)		2 (100)
	Ser531Tryp/Val456Gly 1 (2)		1 (100)
	Gln513Lys 2 (4)		2 (100)
	Leu533Pro 1 (2)		1 (100)
	His516Tyr 1 (2)		1 (100)
	Leu511Pro 1 (2)		0
	No mutation 22 (43)		6 (27)
	No amplification 1 (2)		0
Fluoroquinolones, *gyrA*	Ser95Thr 46 (90)†		
	Asp94Gly 1 (2)		
	Asp94Ala 1 (2)		
	Ala90Val 1 (2)		
	No amplification 2 (4)		
Ethambutol, *embB*	Asp354Ala 3 (6)		1 (33)
	Asp354Ala/Gly406Asp 1 (2)		0
	Met306Val 3 (6)		3 (100)
	Met306Ile 3 (6)		3 (100)
	Gly406Ser 3 (6)		2 (67)
	Gly406Ala 2 (4)		0
	Gly406Cyst 1 (2)		1 (100)
	No mutation 25 (49)		9 (36)
	No amplification 10 (20)		3 (30)
			Not performed
Pyrazinamide, *pncA*‡	Gly113Phe 3 (6)§	G338T and C96T	
	Leu19Arg 2 (4)§	T56G	
	Gly113Phe/Arg121 Leu 1 (2)§	G338T and C96T/G362T	
	Arg121Leu 1(2)§	G362T	
	Gln10Pro 1 (2)	A29C	
	Val7Gly 1 (2)	T20G/G481C	
	Ala161Pro/Val155Ala 1 (2)§	G203A	
	His137Asp/Frameshift 1 (2)§	T464C/insertion C480	
	Tryp68Stop 1 (2)§	C409G	
	Frameshift 1 (2)§	deletionG5	
	No mutation 8 (14)		
	No amplification 30 (59)		
Kanamycin			
rrs		A1401G 4 (57)	3 (75)
		C1443G 3 (43)	1 (33)
		No mutation 35	12 (34)
		No amplification 9	3 (33)
eis		G(−10)A 4 (44)	2 (50)¶
		C(−14)T 1 (11)	1 (100)¶
		C(−15)G 2 (22)	0
		C(−14)G 1 (11)	0
		C(−12)T 1 (11)	0
		No mutation 36	11
		No amplification 6	3 (50)

*Sequencing for *inhA* and *katG* and correlation with isoniazid resistance available in expanded online version of Table 2 (wwwnc.cdc.gov/EID/article/19/10/12-1108-T2.htm).
†Previously demonstrated not to be associated with phenotypic resistance.
‡Excluding 25 silent *pncA* mutations (Ser32Ser most common, n = 14).
§Mutations in *pncA* not previously reported. Conventional susceptibility testing was unavailable for pyrazinamide and the fluoroquinolones.
¶For all 3 mutations of *eis* associated with kanamycin resistance, *rrs* was wild type.

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Clonal Distribution and Virulence of *Campylobacter jejuni* Isolates in Blood

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Campylobacter jejuni bacteria are highly diverse enteropathogens. Seventy-three *C. jejuni* isolates from blood collected in Finland were analyzed by multilocus sequence typing and serum resistance. Approximately half of the isolates belonged to the otherwise uncommon sequence type 677 clonal complex. Isolates of this clonal complex were more resistant than other isolates to human serum.

The most common bacterial enteropathogen in industrialized countries is *Campylobacter jejuni*. This bacterium typically causes watery diarrhea with fever and abdominal pain (*1*,*2*). Complications, such as bacteremia, Guillain-Barré syndrome, and reactive arthritis, might also occur (*3*).

Multilocus sequence typing (MLST) has shown that *C. jejuni* is weakly clonal and highly diverse (*4*,*5*). Several MLST studies have identified particular niches for certain genetically related MLST lineages (*6*,*7*). Thus, MLST is robust in population genetics and source attribution studies.

Susceptibility to human serum varies between different species of *Campylobacter*; *C. fetus* is typically resistant, and *C. jejuni* is believed to be sensitive (*8*). Because serum resistance might contribute to spread of *C. jejuni* in the bloodstream, systemic isolates have been studied for their survival in human serum. However, numbers of isolates studied have been limited, and results compared with those for fecal isolates have not been distinctive (*8*,*9*).

In a recent nationwide study over a 10-year period, we collected blood culture isolates of *C. jejuni* and *C. coli* and obtained clinical features of corresponding bacteremic episodes and characteristics of patients throughout Finland (*10*). Our results showed that patients were moderately young and mostly without any underlying diseases (*10*). In the present study, we characterized *C. jejuni* blood culture isolates with respect to their clonal distribution and serum resistance.

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The Study

The bacterial isolates were collected throughout Finland during 1998–2007 as described (*10*). Of 76 patients described, 3 were excluded because of *C. coli* infections. MLST was performed for 73 *C. jejuni* isolates as described (*11*). BioNumerics version 5.1 software (Applied Maths, Kortrijk, Belgium) was used for sequence assembly. Allele numbers, sequence types (STs), and clonal complexes (CCs) were assigned by using the PubMLST database (*5*). New alleles and STs were submitted to the database.

A serum sensitivity assay was conducted with 73 *C. jejuni* isolates according to a described protocol (*8*). The same pool of serum samples from 10 healthy blood donors was used in all experiments. *C. jejuni* NCTC 11168 and a *C. fetus* isolate from blood were used as control organisms.

All statistical analyses were performed by using Graphpad Prism version 4.03 (Graphpad Software, San Diego, CA, USA) and PASW Statistics version 18 (SPSS Inc., Chicago, IL, USA). The χ^2 test and Fisher exact test were used for comparison of categorical variables. The Mann-Whitney test was used for the comparison of continuous variables. All tests were 2-sided, and $p<0.05$ was considered significant.

A total of 72 *C. jejuni* isolates from blood were successfully typed by MLST; 1 isolate had a mixed MLST pattern. Five isolates were in unassigned STs, and the rest were distributed among 11 CCs (Table). ST-677 CC was the predominant complex: 35 (48%) isolates. Genetic relatedness of these isolates was further confirmed by using pulsed-field gel electrophoresis.

Isolates belonging to ST-677 CC were obtained throughout the 10-year study period. However, bacteremia episodes caused by ST-677 CC isolates were exclusively diagnosed during the seasonal peak during May–August (Figure 1). Of *C. jejuni* blood culture isolates detected during May–August, most (64%) were ST-677 CC. Furthermore, ST-677 CC was the most prevalent complex in 4 geographic regions of Finland.

Susceptibility to human serum varied between *C. jejuni* isolates from different CCs (Figure 2). ST-677 CC isolates were significantly less susceptible to human serum than all other isolates ($p<0.0001$). ST-45 CC isolates were significantly more susceptible to human serum than all other isolates ($p<0.0001$).

Conclusions

We characterized a unique collection of 73 *C. jejuni* isolates from blood obtained during a nationwide study in Finland over a 10-year period. Despite the high population diversity of *C. jejuni*, nearly half of the isolates from blood showed clustering within the ST-677 CC, a rare CC in other countries (*12*,*13*). Furthermore, bacte-

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Table. Distribution of MLST clonal complexes and sequence types among 73 blood culture isolates of *Campylobacter jejuni**

Clonal complex	No. (%)	ST	No.	Characteristics significantly (p<0.05) associated with clonal complex
ST-677	35 (48)	677	27	Serum resistance
		794	8	
ST-45	12 (16)	11	4	Serum sensitivity
		45	3	
		137	2	
		230	2	
		5201	1	
ST-21	10 (14)	50	5	Underlying
		883	2	disease and
		1948	1	longer duration of
		5670	1	hospitalization of
		Uncertain	1	patients
ST-48	2 (3)	38	1	ND
		48	1	
ST-464	2 (3)	464	1	ND
		3140	1	ND
ST-52	1 (1)	52	1	ND
ST-354	1 (1)	3155	1	ND
ST-443	1 (1)	5671	1	ND
ST-460	1 (1)	606	1	ND
ST-508	1 (1)	508	1	ND
ST-1332	1 (1)	1332	1	ND
Unassigned	5 (7)	468	1	ND
		1080	1	ND
		1972	1	ND
		5673	1	ND
		5674	1	ND
Mixed	1 (1)	Uncertain	1	ND

*MLST, multilocus sequence typing; ST, sequence type; ND, none detected.

rial survival in human serum varied greatly. Thus, invasiveness of blood culture isolates could not be solely explained by their serum resistance, although the predominant isolates of ST-677 CC were more serum resistant than other isolates.

C. jejuni has high ST diversity. As of May 2, 2013, a total of 6,564 STs were registered (*5*). In this study, we detected clustering of *C. jejuni* isolates from blood in an uncommon ST-677 CC. Further studies are needed to clarify whether bacterial characteristics might explain this finding.

In our previous study, which included human fecal *C. jejuni* isolates obtained in Finland from the mid-1990s through 2007, which is nearly the same period as in the current nationwide study, 11.7% of the isolates belonged to ST-677 CC (*11*). The 2 most prevalent CCs in that study, ST-45 CC (43.6% of fecal isolates) and ST-21 CC (19.4% of fecal isolates), were detected only among 12 (16%) and 10 (14%) of blood culture isolates, respectively, in the present study.

ST-45 CC and ST-21 CC have been shown to be prevalent in several countries (*4*,*13*). However, our results suggest that these 2 CCs are not common among *C. jejuni* isolates from blood in Finland, which cluster more in the ST-677 CC. On the basis of the present results, we

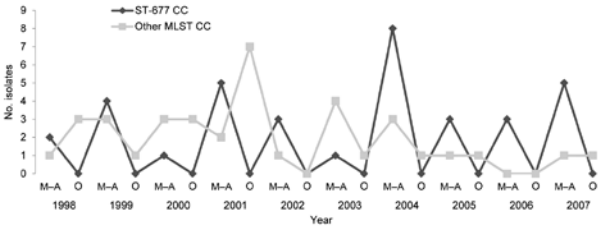

Figure 1. Annual and seasonal distribution of 72 *Camplyobacter jejuni* blood culture isolates belonging either to the ST-677 clonal complex (CC) or to the other multilocus sequence typing (MLST) CCs. One isolate with a mixed multilocus sequence type was not included. *C. jejuni* bacteremia was diagnosed during May–August (M–A) or during any other month of the year (O).

speculate that ST-677 CC might have a special invasive capability or has adapted to the environment in Finland.

In general, complement-mediated killing of serum-susceptible isolates plays a major role in restricting access of pathogens to the bloodstream. However, available information about possible serum sensitivity of *C. jejuni* isolates from blood is scarce (*8*,*9*). In our study of nonselected *C. jejuni* isolates from blood, susceptibility to human serum varied according to MLST CC.

In conclusion, in this nationwide study during a 10-year period in Finland, we found by MLST analysis that half of the bacteremia isolates of *C. jejuni* clustered within an otherwise uncommon ST-677 CC. Whether this finding indicates special adaptation of ST-677 CC to Finland or to the human bloodstream is not clear and needs to be studied. Our findings emphasize the role of using well-defined clinical materials in studies on bacterial pathogenicity and severity of human disease.

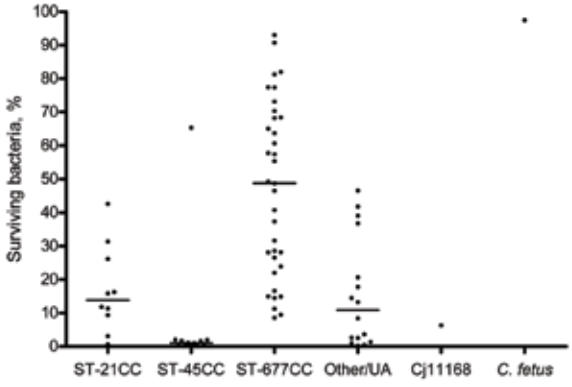

Figure 2. Percentage of surviving bacteria in human serum for 73 blood culture isolates of *Campylobacter jejuni* (Cj), grouped according to major multilocus sequence typing clonal complexes (CCs), and for controls *C. jejuni* Cj11168 and *C. fetus*. Dots indicate mean values for 2–3 experiments. Horizontal lines indicate median values for each CC group. ST, sequence type; UA, unassigned.

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Melioidosis in Traveler from Africa to Spain

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The worldwide epidemiology of melioidosis is changing. We describe a case of acute melioidosis in Spain in a patient who had traveled to Africa. A novel sequence type of *Burkholderia pseudomallei* was identified in this patient. Clinicians should be aware of the possibility of melioidosis in travelers returning from melioidosis-nonendemic regions.

Melioidosis is caused by infection with the gram-negative bacterium *Burkholderia pseudomallei*. Autochthonous cases of melioidosis occur throughout Southeast Asia and northern Australia, but sporadic cases have occurred in other locations, and evidence suggests that the epidemiology of this disease is changing. Cases have been reported in tropical regions of South America, particularly northeastern Brazil (*1*), and other cases have been imported from the Caribbean (*2*). In addition, several cases have been identified in Africa and in travelers returning to European countries from Africa (Table). We report a case of acute melioidosis in a traveler from Africa who was admitted to a hospital in Madrid, Spain.

The Case

A 35-year-old previously healthy woman was admitted to the emergency unit of Ramón y Cajal University Hospital, Madrid, Spain, on March 25, 2011, because of a 4-day fever and arthralgias. She had just returned from an 11-month leisure travel trip through Africa, during which she visited Madagascar and 14 countries in West Africa. At admission, she had fever (39°C) and inflammatory signs on the left ankle. HIV test results were negative, and a chest radiograph showed no abnormalities. The patient was treated with intravenous ceftriaxone (2 g/d); oral doxycycline (100 mg 2×/d) was added the next day.

On the third day, acute progressive dyspnea, cough, hypoxemia, and hypotension developed. A chest and abdominal computed tomography scan showed bilateral lung miliary infiltrates. Bronchoscopy showed bronchial inflammatory signs; mucopurulent secretions were concomitantly observed. Cultures of blood samples taken in the emergency unit were negative, but a gram-negative bacillus was cultured from blood taken on the third day of hospitalization. Gram stain of respiratory specimens taken at bronchoscopy showed a substantial number of polymorphonuclear leukocytes with predominant gram-positive flora. After 48-h incubation at 35 ± 2°C in sheep blood (ambient air) and chocolate agar (5% CO_2), mostly α-hemolytic colonies of streptococci with a few colonies of saprophytic *Neisseria* spp. were cultured. Treatment was changed to intravenous ceftazidime (2 g 4×/d) and oral doxycycline (100 mg 2×/d), and the patient showed rapid clinical improvement. After 3 weeks of treatment, the patient was discharged with maintenance treatment of oral amoxicillin/clavulanic acid (1 g 3×/d) and doxycycline (100 mg 2×/d), later changed to oral cotrimoxazole (1,920 mg/d) for 3 months course. After a year, the patient had no sequelae or relapsing symptoms attributable to melioidosis.

The aerobic bottles of the 2 sets of blood specimens collected from the patient after hospitalization were positive for gram-negative rods with clear, bipolar staining. Subcultures on sheep blood agar plates (35 ± 2°C) yielded a bacillus forming greyish colonies with an intense, earthy odor. The organism was identified as *B. pseudomallei* with a 79.0% probability score by the WIDER system (Fco. Soria-Melguizo, Madrid, Spain); the API 20NE system (bioMérieux, Marcy l'Étoile, France) identified the isolate as *B. pseudomallei* with the numeric profile 1156577 (99.9% certainty). Mass spectrometry (MALDI-TOF MS; Bruker Daltonics, GmbH, Leipzig, Germany) initially identified the isolate as *Burkholderia thailandensis*, likely because of the paucity of entries in the database at that time (*10*).

The full 16S rRNA gene (≈1,500 bp) of the isolate was amplified and sequenced. Nucleotide sequence alignment results were identical to results of a *B. pseudomallei* isolate from GenBank (accession no. AF93059). Susceptibility testing was performed (WIDER system), and results were interpreted according to Clinical and Laboratory Standards Institute *Burkholderia cepacia* guidelines (*11*). The isolate was susceptible (MIC, μg/mL) to ceftazidime (≤1), meropenem (≤2), minocycline (≤2), levofloxacin (≤2), and cotrimoxazole (≤2/38).

The isolate was sent to the National Reference Center for Microbiology (Majadahonda, Spain), where it was labeled BpSp2 and analyzed by a reverse line blot PCR that enables the specific identification of *B. mallei*, *B. pseudomallei*, and *B. thailandensis* (*7*). The isolate was further characterized by multilocus sequence typing (MLST) (*12*); the allele sequence and profile identified were submitted to the public database (http://bpseudomallei.mlst.net) for number assignation, and the obtained sequence type (ST)

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Table. Characteristics of reported cases of human melioidosis with African origin*

Patient age, y/sex	Area visited/ diagnosed	Sample type	Final treatment	Outcome	Clinical risk factors	Possible source	Method of identification†	Reference
64/M	Kenya/Denmark	Blood, urine, sputum	TET+SXT	Recovered	Pulmonary tuberculosis	NR	Serology	(*3*)
12/F	Sierre Leone/The Gambia/	Bony abscesses	CMP+TET	No follow-up	NR	NR	Serology	(*4*)
40/F	Mauritius	Blood	CTX+MDZ	Died	SLE	Rainy season	API 20NE	(*5*)
58/M	Madagascar/La Réunion (France)	Sputum	IMI	Recovered	NR	Unknown	API 20NE, serology, PCR	(*6*)
29/M	The Gambia, Guinea Bissau, Senegal]/Spain	Soft tissue abscesses	CAZ+SXT	Recovered	Hepatitis Diabetes	Rainy season	Vitek II System, API 20NE, PCR	(*7*)
46/F	Nigeria/United Kingdom	Blood	MER	No follow-up	Diabetes	Trip to Nigeria	Chromatography, PCR	(*8*)
60/M	NR/France	Blood	IMI+CIP	Recovered	NR	Unknown	NR	(*9*)
35/F	Madagascar, West Africa/Spain	Blood	CAZ+DOX	Recovered	None	Goat raw milk ingestion	WIDER System, API 20NE, serology, PCR, MLST	This study

*TET, tetracycline; SXT, cotrimoxazole; NR, not reported; CMP, chloramphenicol; CTX, cefotaxime; MDZ, metronidazole; SLE, systemic lupus erythematosus; IMI, imipenem; CAZ, ceftazidime; MER, meropenem; CIP, ciprofloxacin; DOX, doxycycline; MLST, multilocus sequence typing.
†API 20NE, bioMérieux, Marcy l'Étoile, France; Vitek II System, bioMérieux; WIDER system (Fco. Soria-Melguizo, Madrid, Spain).

was compared with other published STs. In addition, the concatenated sequences of the 7-MLST genes (3,399-bp) of the BpSp2 strain and the STs available in the public database were aligned by using ClustalX (www.clustal.org), and neighbor-joining phylogenetic trees were constructed by using MEGA4 software (*13*). *B. pseudomallei* strain NCTC 10276 was used to prepare immunofluorescence assay slides. Two serum samples from melioidosis patients in Thailand were used as positive controls, and a serum sample from a patient in Spain who had bacteremia caused by *B. cepacia* was used as a negative control. A convalescent-phase serum sample from the patient, a sample from her husband, and serial serum samples from 7 hospital workers suspected of potential exposure were analyzed.

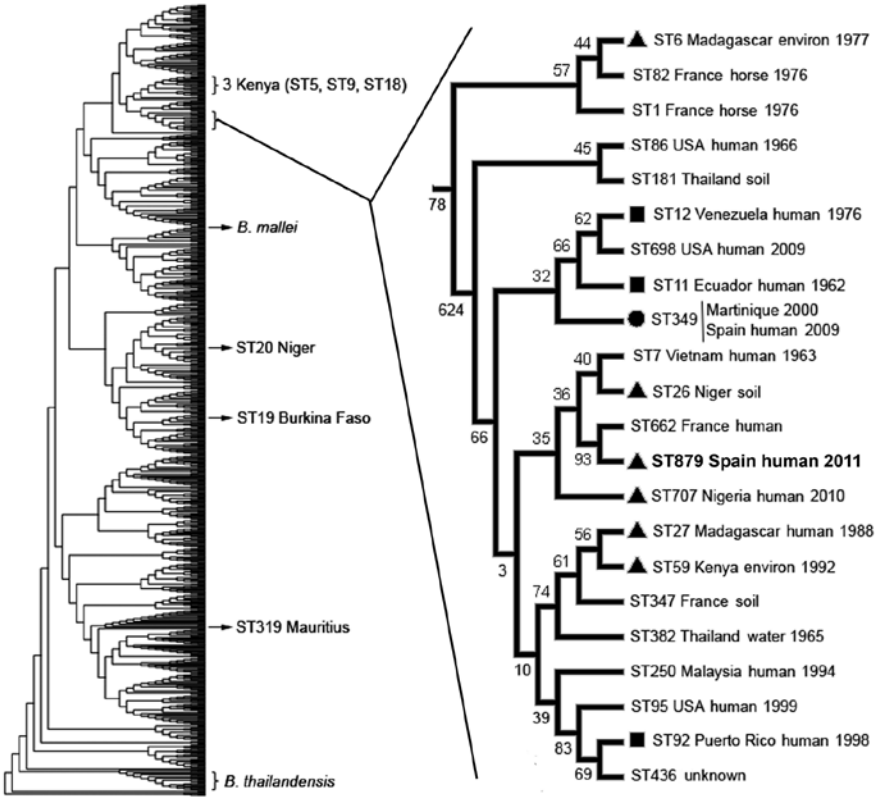

Figure. Phylogenetic position of *Burkholderia pseudomallei* isolate BpSp2, sequence type (ST) 879 (**boldface**), from a patient in Spain who had traveled to Africa. The dendrogram was built by using 852 isolates from the public *B. pseudomallei* database (http://bpseudomallei.mlst.net). The clade in which most STs from Africa, South America, and the Caribbean are located has been enlarged; location, source type, and year collected are indicated for each isolate. Black circle indicates isolates from Africa; black squares indicate isolates from South America or the Caribbean; black triangles indicate ST349 from Martinique and the first patient described in Spain (*7*). Location of other STs from Africa, *B. mallei*, and *B. thailandensis* are also indicated. Environ, environment.

The BpSp2 isolate was confirmed as *B. pseudomallei* by PCR by using the specific probe for this pathogen (data not shown). MLST showed a new allelic profile for this strain, including a new allele for *ndh* that was numbered 39. This ST was identified as 879 (1, 1, 10, 2, 6, 1, 39) in the database and is close to the human ST349 (Martinique and Spain) (*7*), ST7 (Vietnam), and ST662 (France). These STs, however, showed 2 different loci compared with ST879, which grouped in the same clade with ST662 and ST7 but also with ST26 (Niger) and ST707 (Nigeria) (Figure). Other than ST29, ST20, and ST319 (Burkina Faso, Niger, and Mauritius, respectively), the isolates from Africa grouped in the same clade with the STs from South America and the Caribbean (Figure).

The patient's serum showed an IgG titer of 640; IgM was negative. The patient's husband and the laboratory workers were negative for IgG and IgM, except that in 1 of the workers, a titer of 40 for IgM was detected, but no seroconversion occurred.

Conclusions

Although Africa is not considered a melioidosis-endemic zone, melioidosis in humans has been reported; melioidosis was confirmed in a traveler returning from Africa to Spain in 2009 (*7*). For the patient we report, common predisposing factors for melioidosis were not observed, and an inoculation event or definite exposure to contaminated soil or water were not identified. The patient had consumed raw, nonpasteurized goat milk, and *B. pseudomallei* has been isolated from this food (*14*), but this possible link was not conclusive.

Confirmatory diagnosis of *B. pseudomallei* infection is isolation of the pathogen from clinical samples of patients, but the identification of *B. pseudomallei* can be elusive because automated systems used in clinical microbiology laboratories may misidentify it as *B. cepacia* (*15*). However, API 20NE has proven to give accurate identification, as has mass spectrometry with an extensive database support. Despite high variability among the *B. pseudomallei* isolates, most of the STs from Africa grouped in the same clade and seemed to be phylogenetically related, particularly with strains from South America and the Caribbean. The phylogenetic analysis strongly suggests that BpSp2 has an African origin.

In conclusion, because of difficulties in diagnosis, cases of melioidosis outside areas to which it is endemic may be more common than realized. An increase in nongovernment organization and medical cooperation programs in Africa, as well as leisure travelers moving to and from Africa, may have resulted in increased frequency of some infections, such as imported melioidosis. These findings require clinicians to recognize the disease and microbiologists to identify the causative agent.

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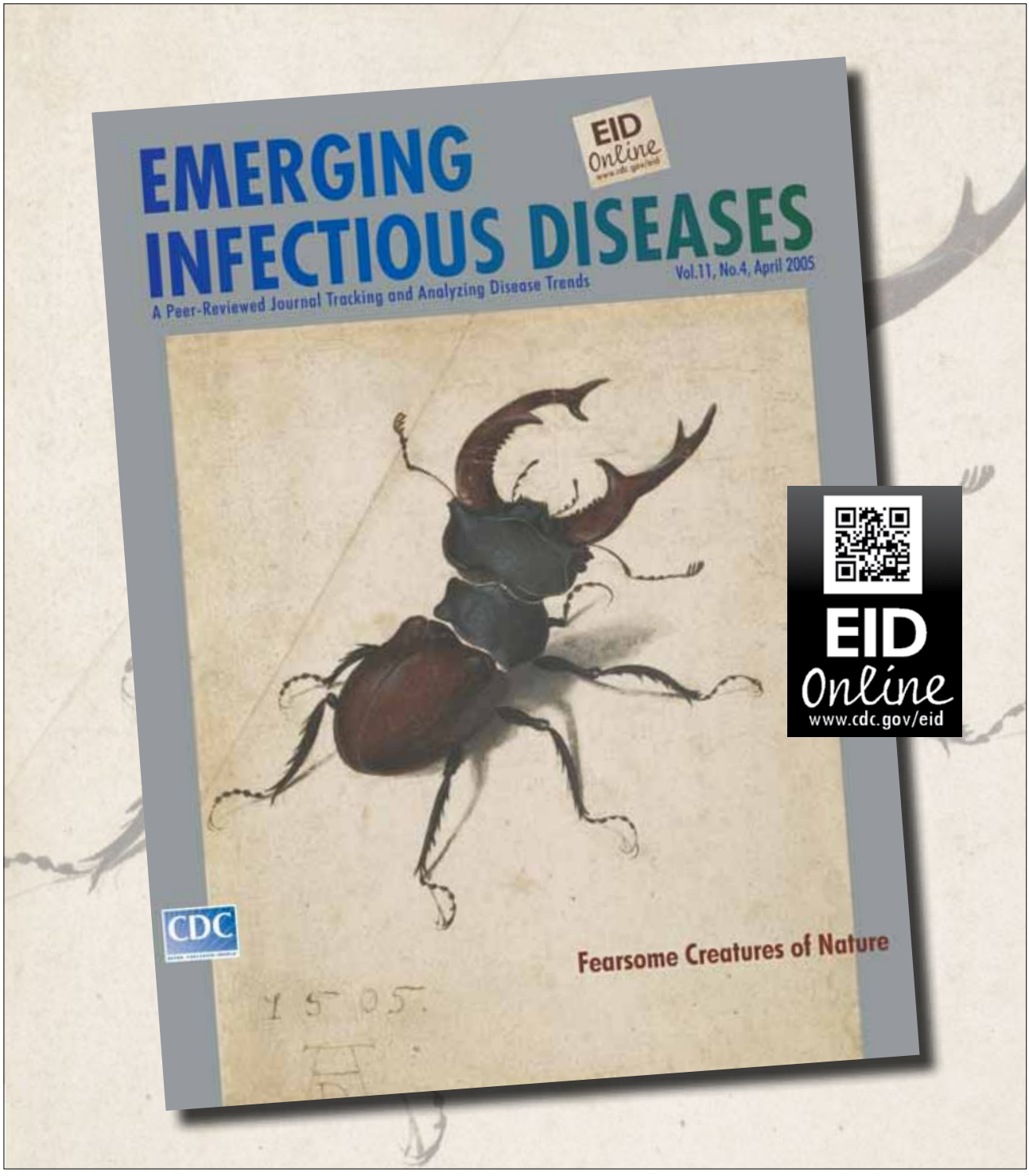

Declining Influenza Vaccination Coverage among Nurses, Hong Kong, 2006–2012

Shui Shan Lee, Ngai Sze Wong, and Sing Lee

Seasonal influenza vaccination of nurses in Hong Kong fell from 57% in 2005 to 24% in 2012, paralleling concern for adverse reactions associated with vaccination. Decreased acceptance of vaccination was most prominent among nurses who had less work experience and more frequent contact with patients.

Despite the moderate effectiveness of influenza vaccination against seasonal infections (*1*), vaccination remains a key prevention strategy for enhancing population preparedness. Vaccination of health care workers serves a dual purpose: self-protection and reduction of transmission in health care settings. Worldwide, vaccination coverage among health care workers is extremely variable: 20%–40% in western Europe (*2*), 16%–60% in Australia (*3*), 63.5% in the United States (*4*), and 30% in Hong Kong (*5*). The main problems with vaccination studies are their incomparability and the changing patterns over time. To determine patterns in Hong Kong, we investigated an 8-year trend of vaccination coverage among nurses.

The Study

In Hong Kong, free yearly influenza vaccination has been offered to all health care workers in the public service since 2003, in the aftermath of severe acute respiratory syndrome. In collaboration with 3 local nurses associations, we administered postal surveys every 1–2 years, focusing on vaccination of nurses and the reasons for their acceptance or decline. The surveys were conducted in 2006, 2007, 2009, 2011, and 2012; during each of these 5 years, 2,500–3,000 questionnaires in the Chinese language were delivered by postal service. Coverage of the surveys ranged from 26% to 38% (Table 1). The surveys included core questions about whether respondents had been vaccinated before the past winter season and whether they planned to be vaccinated in the next year. An incentive, in the form of a book or cash coupon for 25 Hong Kong dollars (1 US$ = 7.8 Hong Kong dollars), was offered to respondents who returned a completed survey form before the end of a 1-month period. Approval of the Survey and Behavioral Research Ethics Committee of the Chinese University of Hong Kong was obtained. Snapshot results of the survey in 2006, 2009, and 2011 have been published (*6*–*8*).

In Hong Kong, influenza vaccination is administered from October through February. In each of the years from 2005 through 2012, the vaccination rate for nurses before the previous winter season was captured. In 2007 and 2012, the percentage of nurses intending to be vaccinated was collected because surveys had not been organized in time to record the actual rate in those years. In 2005, the vaccination rate was 56.8%. It ranged from 50% to 60% irrespective of age, sex, professional rank (registered nurse or enrolled nurse), amount of work experience, and intensity of patient contact (always, sometimes, seldom, or never) (Table 2). A steady decline in vaccination of nurses was observed, more prominently after 2009; the prediction for 2012 was for vaccination of <25% of nurses (Figure). The rates have diversified between subgroups of nurses, despite similarity by sex and professional rank (Table 2). The largest decline in vaccination occurred among nurses with less work experience. Whereas 51% of those with ≤10 years' experience were vaccinated in 2005, only 10% were vaccinated in 2012. Surprisingly, nurses always in contact with patients were less inclined to be vaccinated; during this period, the rate of vaccination coverage fell from 54% to 23%. Similarly, vaccination rates among those seldom or never in contact with patients also declined, from ≈60% in 2005 to ≈25% in 2010. Thereafter, the vaccination rate increased to ≈40% in those seldom in contact with patients but continued to fall to ≈20% among nurses not needing to work with patients.

Overall, self-protection was the main reason for receiving seasonal influenza vaccination, increasing from 74% in 2006 to >90% in 2011 and 2012 (Table 1). The proportion of nurses who indicated that "protecting others" was their reason for vaccination almost doubled since 2006, reflecting nurses' improved understanding of infection control principles. Lack of a work requirement for vaccination was not a main reason for not getting vaccinated because vaccination is not mandatory in Hong Kong. For nurses who refused to be vaccinated, the main reason was "concern for side effects," as indicated by 42% in 2006, 70% in 2011, and 56% in 2012. A smaller proportion of respondents indicated refusal to be vaccinated because of "ineffectiveness for self-protection" and "ineffectiveness for protecting others."

Apparently, attitudes of nurses could be influenced by prevailing epidemic situations locally and internationally. Elsewhere, increased vaccination during the 2009

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Table 1. General characteristics of questionnaire about influenza vaccination and nurses' responses

Characteristic	Date of survey				
	2006 Sep–Oct	2007 Oct–Nov	2009 July–Aug	2011 Mar–Apr	2012 Feb–Mar
General					
No. questionnaires delivered	2,825	2,929	2,929	2,494	2,492
Response rate, %	38.2	26.9	27.7	28.4	26.6
Total no. respondents	903	739	779	477	651
Male, %	11.7	14.1	14.2	17.6	15.1
Reasons for getting vaccinated, no. respondents*	510	339	306	127	161
Self-protection, %	74	88	87	94	91
Protecting others, %	32	58	53	71	67
Work requirement, %	38	55	62	39	33
Reasons for not getting vaccinated,* no. respondents	377	397	451	350	490
Concern about side effects, %	42	55	62	70	56
Ineffectiveness for self-protection, %	50	57*	51	45	50
Ineffectiveness for protecting others, %	9	22	14	13	23
Lack of work requirement, %	7	9	6	6	5

*A multiple-choice list was provided for respondents. Only common reasons specifically chosen by respondents in each survey are included in the table, in order of their preferences. Less than 5% chose "other" as their reason for getting vaccinated, whereas ≈20% chose "other" as their reason for not getting vaccinated.

influenza A(H1N1) pandemic has been reported (*9*), a phenomenon similar to that in Hong Kong after the 2003 outbreak of severe acute respiratory syndrome (*6*). Our serial surveys indicated a peak rate of 57% in 2005, which fell to 46% in the next year. For some experienced nurses (>20 years' experience, ages >45 years), vaccination coverage rate was ≈50% in 2006, when outbreaks of infectious diseases, notably avian influenza, became evident in neighboring mainland China (*10*). The gradual decline of vaccination coverage paralleled the relatively quiet years; it followed the usual bimodal seasonal peaks until influenza A(H1N1)pdm09 virus emerged. Before this new epidemic, the vaccination rate was 40%, comparable to the rate determined by another study of health care workers in Hong Kong (*11*). In retrospect, by the time seasonal vaccination was offered toward the end of 2009, the first

Table 2. Influenza vaccination rates and characteristics of nurses, Hong Kong, 2005–2012

Characteristic	Year							
	2005	2006	2007*	2008	2009†	2010	2011	2012*
Total sample size	903	739	739	779	477	477	651	639
Winter influenza season	Mar–Jul‡	Feb–Apr	Feb–May	Feb–Mar	Jan–Mar	Mar-–May	Jan–Mar	Jan-Mar
Predominant influenza virus strain	A(H3N2)	A(H1N1)	A(H3N2)	A(H1N1)	A(H1N1)	B	A(H1N1) pdm09	B
Overall vaccination rate, % (95% CI)	56.8 (53.6–60.0)	46.0 (42.4–49.6)	41.1 (37.6–44.7)	39.8 (36.3–43.2)	39.8 (35.4–44.2)	26.6 (22.6–30.6)	24.7 (21.4–28.1)	24.4 (21.1–27.8)
Vaccination rate by subgroup, no. (%)								
Sex								
M	106 (58)	104 (44)	104 (42)	111 (44)	84 (39)	84 (33)	98 (31)	95 (29)
F	797 (57)	635 (46)	635 (41)	668 (39)	393 (40)	393 (25)	553 (24)	544 (24)
Age, y								
≤35	225 (52)	104 (38)	104 (30)	85 (44)	22 (36)	22 (23)	88 (15)	86 (16)
36–45	422 (59)	363 (45)	363 (40)	318 (36)	138 (32)	138 (28)	159 (25)	155 (26)
>45	256 (57)	272 (50)	272 (47)	376 (42)	317 (44)	317 (26)	404 (27)	398 (25)
Professional rank								
Registered nurse§	670 (57)	558 (46)	558 (41)	636 (40)	397 (40)	397 (27)	537 (24)	527 (24)
Enrolled nurse#	203 (55)	163 (44)	163 (39)	124 (41)	77 (38)	77 (22)	96 (27)	94 (24)
Working experience								
≤10 y	146 (51)	57 (42)	57 (35)	71 (44)	21 (33)	21 (19)	80 (14)	78 (10)
11–20 y	373 (58)	275 (40)	27 (35)	240 (37)	103 (30)	103 (22)	151 (22)	147 (23)
>20 y	356 (59)	385 (51)	385 (46)	452 (41)	353 (43)	353 (28)	416 (28)	410 (28)
Patient contact								
Always	679 (54)	546 (42)	546 (37)	521 (39)	311 (37)	311 (26)	447 (23)	442 (23)
Sometimes	79 (59)	62 (60)	62 (50)	76 (37)	41 (44)	41 (34)	64 (33)	62 (27)
Seldom	79 (68)	71 (55)	71 (55)	83 (49)	50 (44)	50 (24)	51 (39)	47 (45)
Never	59 (66)	57 (53)	57 (49)	93 (40)	7 (46)	72 (26)	87 (17)	86 (17)

*Planned vaccination as indicated by nurses in the preceding year.
†Vaccination history based on nurses' recall the following year.
‡Winter and summer seasons merged, giving an extended period of influenza activities.
§Completed 3 y of training in a recognized school and holds a degree/diploma in nursing or equivalent.
#Completed 2 y of training in a recognized training school under a hospital-based program.

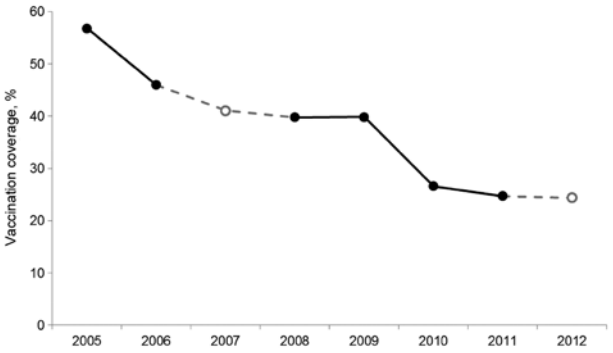

Figure. Rates of influenza vaccination among nurses, before the respective winter seasons, Hong Kong, 2005–2012. Closed circles indicate data based on nurses' recall a year later; open circles indicate data based on nurses' plans to get vaccinated the next year.

wave of A(H1N1)pdm09 infection had already subsided. Vaccination coverage in the next year fell to 30%. In addition to the seasonal vaccine, a new monovalent vaccine against A(H1N1)pdm09 virus was introduced at the same year end. The new vaccine was accepted by only 14.2% of the nurses, as indicated by our 2011 survey results (*8*). The association of A(H1N1)pdm2009 vaccine with Guillain-Barré syndrome was a deterrent to vaccination, despite the minimal increased risk (1 case/1 million vaccinees) (*12*), especially in light of the mild nature of influenza disease caused by A(H1N1)pdm09 virus. The incorporation of A(H1N1)pdm09 virus in the trivalent vaccine at the end of 2010 could be one reason for the precipitous fall in vaccination coverage (*8*).

Our study had some limitations. The small sample size and focus on nurses, most of whom were in the public service, imply that generalization of the results to all health care workers would need to be made with caution. Also, repeated administration of the same survey might have led some respondents to choose a desirable rather than an appropriate answer. In addition, although the profile of respondents has remained similar, minor changes in characteristics might have occurred, making interpretation of longitudinal patterns difficult.

Conclusions

Influenza vaccination among health care workers is a complex issue. Our results suggest that whereas self-protection was the main reason for choosing seasonal influenza vaccination, concern about side effects was the main reason for refusing to be vaccinated. A balance of these 2 factors varies with time and is associated with the epidemic condition perceived by the nurses in this study. To enhance societal preparedness, influenza vaccination coverage among health care workers should be maintained at a high level. Those who are at highest risk for virus transmission, i.e., those on the front line and in frequent contact with patients, should be the primary focus of vaccination campaigns. The temporal trend of health care workers' acceptance of seasonal influenza vaccination was evidently not uniform. For future vaccination strategies, measures for improving vaccination coverage should be tailored to the needs of subcategories of health care workers, including nurses, as defined by their potential for virus transmission.

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Hepatitis E Virus among Persons Who Inject Drugs, San Diego, California, USA, 2009–2010

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Data about prevalence of hepatitis E virus infection in persons who inject drugs are limited. Among 18–40-year-old persons who inject drugs in California, USA, prevalence of antibodies against hepatitis E virus was 2.7%. This prevalence was associated with age but not with homelessness, incarceration, or high-risk sexual behavior.

Serologic evidence of hepatitis E virus (HEV) infection (i.e., IgG against HEV) in the United States has been reported to be ≤21% on the basis of national estimates during 1988–1994 (*1*). Among marginalized populations, such as persons who inject drugs (PWID) and homeless or incarcerated persons, HEV infection ranges from 5% to 23%, although data have been limited for these groups (*2–6*). We determined the seroprevalence of and factors associated with IgG against HEV among 18–40-year-old PWID in San Diego, California, USA.

The Study

Methods for the Study to Assess Hepatitis C Risk have been summarized (*7*). In brief, during March 2009–June 2010, persons 18–40 years of age who were residents of San Diego County, California, and who had injected drugs in the previous 6 months were recruited to participate in this study. Eligibility screening and acquisition of informed consent for potential participants were followed by a behavioral risk assessment and serologic testing.

Data collected included participant demographics, substance use, injection practices, diagnosis with sexually transmitted infections, exchange of sex for money, homelessness, travel to Mexico, and HIV status. Serologic testing included detection of antibodies against hepatitis A virus (HAV), hepatitis B virus core antigen, and hepatitis C virus (HCV) by using the VITROS Immunodiagnostic System (Ortho Clinical Diagnostics, Rochester, NY, USA), and IgG against HEV by using a commercial assay (DSI, Saronno, Italy).

We performed a comparative analysis of all persons on the basis of their status for IgG against HEV by using demographics, seropositivity for other viral hepatitides, travel to Mexico, history of incarceration, homelessness, HIV status, and high-risk sexual behavior. We used bivariate logistic regression to calculate odds ratios; 95% CIs; and p values, which were set at 0.05 to determine significance for factors associated with HEV prevalence. All data were analyzed by using SAS version 9.2 (SAS Institute, Cary, NC, USA).

Of 508 PWID, 72% were men, their mean age was 29 years (range 18–40 years); and 62% were white. Fourteen (2.7%) persons had IgG against HEV; none of these persons were positive for HEV RNA by PCR (all were negative for IgM against HEV). Of the 14 persons with IgG against HEV, 11 (79%) were men; their mean age was 33.4 years (range 30–36 years); and 57% were white (Table). Relative to participants <30 years of age, persons ≥30 years of age were more likely to be positive for IgG against HEV (odds ratio 3.61, 95% CI 1.31–9.94). Travel history and presence of antibodies against HAV, hepatitis B virus, or HCV were not associated with presence of antibody against HEV. Bivariate logistic regression showed that there was no association between presence of IgG against HEV and a history of incarceration, sharing of injection drug equipment, homelessness, high-risk sexual behavior, and HIV status.

Conclusions

We found an overall HEV seroprevalence of 2.7% in young PWID in the United States. This seroprevalence was higher among participants ≥30 years of age than in participants <30 years of age. Variables typically associated with HCV/HIV transmission (i.e., high-risk sexual behavior, incarceration, or sharing of injection drug use equipment) were not associated with presence of antibodies against HEV. These findings were consistent with results of a study that found no association between antibodies against HEV and co-infection with other hepatitis viruses or sharing of drug paraphernalia (*2*).

Because of the common mode of fecal–oral transmission of HAV and HEV, other studies have also investigated an association between HAV and HEV infections, but results have been inconclusive (*1,5,6*). As in previous studies, we found an association of presence of antibodies against HEV and age (*1,6*). Higher prevalence among older PWID suggests that there may be age-related

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Table. Prevalence of IgG against hepatitis E virus among persons who inject drugs, San Diego, California, USA, 2010*

Characteristic	Positive for IgG against HEV, n = 14	Negative for antibody against HEV, n = 494†	p value
Sex			
M	11 (79)	357 (72)	0.707
F	3 (21)	137 (28)	ND
Mean age, y (95% CI)	33.4 (30.1–36.6)	28.5 (27.9–29.0)	<0.003
Age ≥30 y	9 (64)	170 (34)	0.013
Race			0.776
White	8 (57)	272 (55)	ND
Black	2 (14)	34 (7)	ND
Hispanic	2 (14)	137 (28)	ND
Other	1 (7)	15 (3)	ND
Homeless	11 (79)	287 (58)	0.326
History of incarceration	12 (86)	376 (76)	0.263
Sharing any drug injecting equipment	8 (57)	354 (72)	0.587
Diagnosis of sexually transmitted infection†	1 (7)	93 (19)	0.443
Exchange of sex for money	5 (36)	142 (29)	0.308
Travel to Mexico	10 (71)	314 (64)	0.373
Antibody against HAV	3 (21)	190 (38)	0.504
Antibody against HBc	3 (21)	201 (41)	0.138
Antibody against HCV	3 (21)	128 (26)	0.351
HIV positive	1 (7)	21 (4)	0.751

*Values are no. (%) unless otherwise indicated. ND, not determined; HAV, hepatitis A virus; HBc, hepatitis B core antigen; HCV, hepatitis C virus.
†Totals may not equal 100% because of missing data.

exposures independent of injection drug use that increases the likelihood of HEV infection. This birth cohort effect has been seen in other low-prevalence countries, such as Denmark (*8*), and decreased possible exposure may help explain the lower prevalence rates in our study.

This study had a few limitations. Our small sample size reduced the potential to detect significant differences between HEV-negative and HEV-positive persons. In addition, we did not have information about other exposures that have been associated with HEV infection, including particular dietary or zoonotic exposures or history of travel to a country to which HEV is endemic. Therefore, the potential effect of these exposures cannot be assessed. Information about HEV genotype was not available for seropositive persons, which might have provided clues as to the mechanism of exposure. Lower prevalence estimates may also reflect the fact that our population only included persons 18–40 years of age. Previous data have suggested that increasing age is associated with higher HEV positivity (*6*), particularly in countries in which prevalence is low and infection is caused mainly by HEV genotype 3 (*9*). Although our data cannot be generalized to the US population, seroprevalence in this study appears to be low, which is similar to time trends in the general population of other low-prevalence areas (*8*).

Variability in assay types used may account for discrepancies seen with previous seroprevalence studies of HEV. In a study evaluating the performance and concordance between various assays for detection of IgG against HEV available at the time, overall concordance ranged from 49% to 94% (median 69%), and concordance among reactive serum samples ranged from 0% to 89% (median 32%) (*10*).

Evaluation of the performance characteristics and concordance of currently available assays for detection of antibodies against HEV, including the assay used in this study, remains to be determined. Overall, our data showed an increase in antibodies against HEV for PWID ≥30 years of age and no other association with other reported risk factors. Future research is needed to explore other marginalized populations in HEV-endemic areas to determine whether there are other risk factors that have not been identified in low-prevalence areas.

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Persistent Human Cosavirus Infection in Lung Transplant Recipient, Italy

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Human cosavirus is a novel picornavirus recently identified in feces from children in southern Asia. We report infection with human cosavirus in a patient in the Mediterranean area. The patient was an adult double lung transplant recipient who had chronic diarrhea associated with persistent infection with human cosavirus.

In 2008, a new virus was detected in fecal samples from children with nonpolio acute flaccid paralysis (*1*). The genome structure of the new virus was closely related to those in the family *Picornaviridae*, but phylogenetic analysis showed that it diverged from known picornaviruses. Thus, a new genus of picornaviruses was proposed: *Human Cosavirus* (common stool-associated virus; HCoSV).

Prevalence of HCoSV varies according to patient age, geographic area, and exposure to enteric viruses in general; it has been identified in fecal samples of children with acute flaccid paralysis in Pakistan and Afghanistan, in healthy children, and in an adult patient in the United Kingdom (*1*). In a study by Kapoor et al., HCoSV strains were classified into 4 species (A, B, D, E). HCoSV was also found in a child with acute diarrhea in Australia (*2*) and, subsequently, was detected in China, Thailand, and Brazil. In China, 3.2% of hospitalized and 1.6% of healthy children were HCoSV positive, and all virus strains belonged to species A (*3*); in Thailand, HCoSV was detected in an adult patient but not in children with acute diarrhea (*4*); and in Brazil, the percentage of HCoV-positive symptomatic children (3.6%) was comparable to that reported in China; however, in 2 groups of asymptomatic children from whom samples were collected at different periods, HCoSV was detected in highly divergent percentages (6.5% vs. 49.2%) (*5*). In Brazil, HCoSV was also detected in an HIV-positive adult patient with acute gastroenteritis, and ≈75% of symptomatic HCoSV-positive patients were co-infected with other gastroenteric viruses. Kapusinszky et al. recently conducted phylogenetic analysis of viral protein (VP) 3–VP1 genes, which revealed greater genetic variability of HCoSV strains, and proposed splitting species A into 24 species (A1–A24), D into 5 species (D1–D5), E into 2 species (E1–E2), and classifying F as 1 species (*6*).

We report a case of HCoSV infection in an immunocompromised woman in northern Italy and the results of retrospective HCoSV testing of 689 stored fecal samples from hospitalized patients with gastrointestinal signs and symptoms. The study was performed according to guidelines of the institutional review board of the Fondazione Istituto Di Ricovero e Cura a Carattere Scientifico Policlinico, San Matteo, Pavia, Italy, on the use of biological specimens for scientific purposes in keeping with Italian law (Article 13 D.Lgs 196/2003) and after having obtained written informed consent from the patient.

The Study

In 2003, a 43-year-old white woman who had undergone a pulmonary lobectomy during childhood underwent bilateral lung transplantation because of severe chronic respiratory failure caused by bilateral bronchiectasis. The posttransplant period was complicated by bronchiolitis obliterans syndrome stage 1, with episodes of acute respiratory distress. The immunosuppressive regimen included tacrolimus (through level 10–15 ng/mL), low-dose steroids (0.10 mg/kg/day), and monthly courses of extracorporeal photopheresis that were initiated after a 3-month course of low-dose azythromycin (discontinued because of lack of efficacy).

In January 2012, the patient's respiratory condition worsened and she was hospitalized. She had cough and fever (38°C), abdominal cramps, and diarrhea (6–10 bowel movements/day, liquid but not bloody). Routine fecal examination and cultures were negative for common gastrointestinal bacteria (*Mycobacteria*, *Salmonella*, *Shigella*, and *Campylobacter* spp.), parasites (protozoa and helminths), and viruses (astrovirus, norovirus, rotavirus, adenovirus). Because of the patient's persistent gastrointestinal symptoms and a positive fecal calprotectin result, a colonoscopy was performed; it revealed inflamed cecal mucosa without ulcerative lesions. Histologic examination of colonic biopsy samples showed mild, chronic, interstitial inflammation, including eosinophils and lymphoid microaggregates. Therefore, fecal samples were examined for less common gastroenteric viral agents—rhinovirus, bocavirus, enterovirus, parechovirus, coronavirus, sapovirus, aichivirus and HCoSV—by using conventional and real-time PCR or nested reverse transcription PCR (RT-PCR) (*1*,*7*–*14*). Samples were negative for all gastrointestinal viruses searched for except for HCoSV. This virus was detected by a nested

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RT-PCR targeting the 5′ untranslated region (UTR) (*1*) and quantified on the basis of serial $\log_{10}$ dilution of extracted nucleic acid estimating the presence of 10^7 RNA copies/mL in fecal samples. The 316-bp 5′ UTR amplicon was sequenced by using an ABI Prism 3100 DNA automatic sequencer (Applied Biosystems, Foster City, CA, USA), assembled by Sequencer software, version 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA), and compared with available HCoSV sequences with the BLAST program (http://blast.ncbi.nlm.nih.gov). Nucleotide identity with the HCoSV species D (species D1) (GenBank accession no. FJ438908) was 93%.

Because the 5′ UTR is not sufficiently discriminatory for typing, we sequenced the highly variable region encompassing VP3–VP1 genes, obtained according to a published protocol (*6*). Phylogenetic analysis, performed with the maximum-likelihood method with parameters selected by the Model Selection tool in MEGA version 5.0 (*15*), showed that the HCoSV VP3–VP1 sequence from Italy clustered with species E strains found in Australia and Bolivia and recombinant D/E strains circulating in Nigeria. Although homology was greatest (97% nt and 81% aa identity) with a strain from Australia (GenBank accession no. FJ555055) (Figure), the hypothesis that this HCoSV strain might be a D/E recombinant strain (*6*) cannot be excluded because the recombination breakpoint between VP1 and the 2A gene was not investigated.

To define the circulation of HCoSV in northern Italy, we retrospectively tested 689 stored fecal samples for HCoSV positivity by using the same nested RT-PCR (*1*). The samples had been collected during April 2011–April 2012 from patients hospitalized at our hospital with gastrointestinal signs and symptoms. Of these 689 patients, 333 (48.3%) were adults (230 [69.0%] immunocompetent and 103 [31.0%] immunocompromised) and 356 (51.7%) were children (275 [77.2%] immunocompetent and 81 [22.8%] immunocompromised). Surprisingly, none of these samples were positive for HCoSV.

In the absence of specific therapy, the patient reported here underwent parenteral rehydration and nutritional supplementation; her general condition improved progressively, and her bowel movements were reduced to 2–3 per day, enabling her to be discharged within a month. However, her gastrointestinal symptoms persisted, and 3 fecal samples collected at 4, 6, and 7 months after discharge were positive for the same HCoSV strain (at a high titer of 10^5–10^7 virus RNA copies/mL feces) but negative for other viruses, bacteria, and parasites.

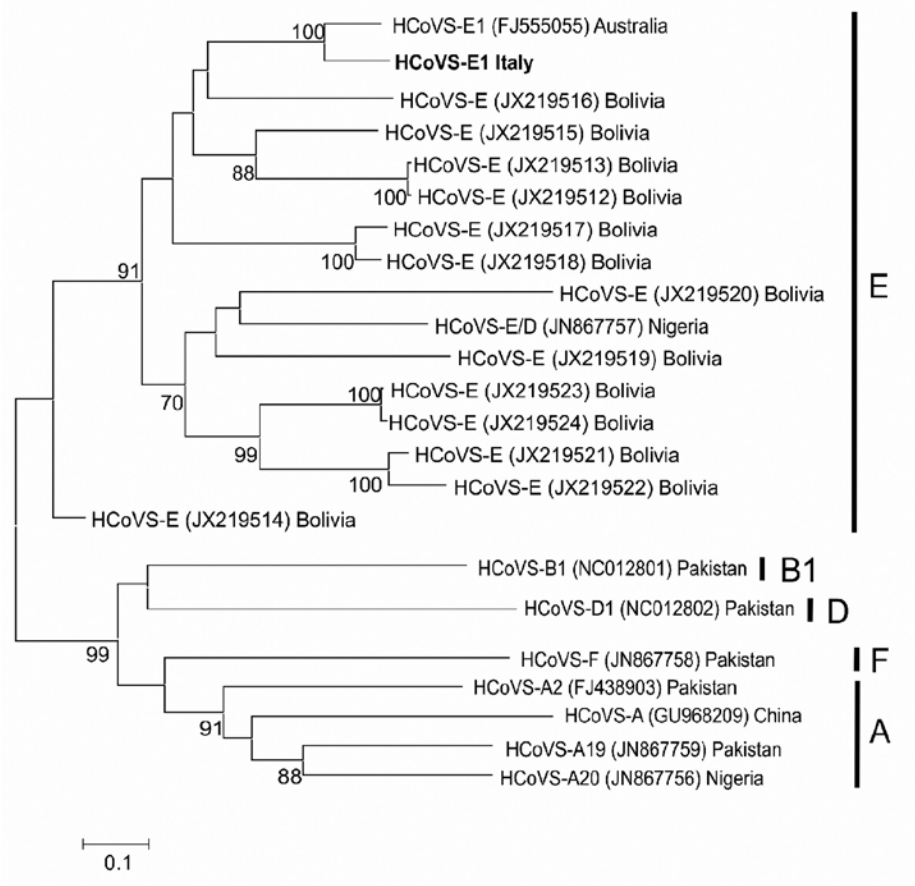

Figure. Maximum-likelihood tree based on viral protein (VP) 3–VP1 sequences. Bootstrap values were set for 1,000 repetitions and were placed over each main node of the tree. Bootstrap values <70% are not shown. The strain from the lung transplant recipient with chronic diarrhea, in Italy, is indicated in **boldface**. Reference strains from GenBank, and their accession numbers, are shown. Letters in the right-hand column indicate virus species. Scale bar indicates nucleotide substitutions per site. HCoVS, human cosavirus.

Conclusions

We reported identification of HCoSV in the Mediterranean area. The infection was detected in 1 of 689 patients with gastrointestinal symptoms who were hospitalized and tested during the same period. Unique to this patient were the prolonged immunosuppressive therapy and the chronic gastrointestinal symptoms with persistently HCoSV-positive fecal samples. The persistence of symptoms and virus are additional new findings that might be associated with the patient's immune impairment.

The source of infection has not been determined. However, the patient denied domestic or international travel and close contact with persons returning from travel. Because no other HCoSV infection was detected in patients hospitalized at the same institution during the same period, we hypothesize that the infection was acquired in the patient's community before her hospitalization. The genetic similarity with E strains circulating in South America, Australia, and Central Africa suggest local acquisition of an imported infection. However, additional evidence is required before excluding the potential autochthonous circulation of HCoSV in northern Italy.

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New Clonal Strain of *Candida auris*, Delhi, India

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A new clonal strain of *Candida auris* is an emerging etiologic agent of fungemia in Delhi, India. In 12 patients in 2 hospitals, it was resistant to fluconazole and genotypically distinct from isolates from South Korea and Japan, as revealed by M13 and amplified fragment length polymorphism typing.

In 2009, yeast isolates from the external ear canal of a Japanese patient were identified as a new species, *Candida auris*, on the basis of sequence analysis of the nuclear rRNA gene. The species is phylogenetically related to *C. haemulonii* (*1*). In the same year, 15 isolates of *C. auris* were reported from otitis media patients in South Korea (*2*). Genotyping of these isolates revealed a clonal origin (*3*). In addition, in 3 patients, persistent fungemia caused by *C. auris* was resistant to fluconazole; 2 of these patients died (*4*). Fungemia caused by *C. auris* seems to be extremely rare, however. We report a series of fungemia cases caused by a new clonal strain of this emerging pathogen, involving 12 inpatients from 2 hospitals in Delhi, India.

The Study

We characterized 12 bloodstream *C. auris* isolates, originating from an equal number of patients, collected during 2009–2011 at 2 hospitals (a tertiary care general hospital and a pediatric center) in Delhi, India. The controls included reference strains of *C. auris* from South Korea (CBS12372, CBS12373) and Japan (JCM15448, DSMZ21092), *C. haemulonii* (CBS7801–7802, CBS5149, CBS5150), *C. pseudohaemulonii* (KCTC1787, CBS10004, JCM12453), and *C. duobushaemulonii* (CBS7798–7800, CBS9754).

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The yeast isolates were oval without pseudohyphae and germ tube formation. They appeared pink on CHROMagar Candida medium (Difco, Becton Dickinson, Baltimore, MD, USA) and grew at 37°C and 42°C. VITEK2 (bioMérieux, Marcy I'Etoile, France) misidentified 10 isolates as *C. haemulonii* and 2 as *C. famata*. Similarly, API20C (bioMérieux) also misidentified them as *C. sake*. *C. auris* isolates from India assimilated *N*-acetylglucosamine (NAG) in contrast to the isolates from Japan and South Korea.

Molecular identification was done by sequencing internal transcribed spacer (ITS) and D1/D2 regions (*5*–*7*). ITS sequences (GenBank accession nos. KC692039–KC692050) of our isolates showed 100% homology with an unrelated *C. auris* isolate (GenBank accession no. HE797773) and 98% identity with isolates from Japan and South Korea (GenBank accession nos. AB375772 and EU884189). Also, large ribosomal subunit sequences (GenBank accession nos. KC692053–KC692064) of the India isolates showed 100% homology with an unrelated *C. auris* isolate (GenBank accession no. HE797774) and 98%–100% identity with isolates from Japan and South Korea (accession nos. AB375773 and EU881960). ITS and large ribosomal subunit sequences of the isolates in our study showed 85%–98% similarity with the closest *C. haemulonii* complex species. These isolates have been deposited at the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (accession nos. CBS12766–CB12777).

M13 PCR fingerprinting analysis was performed as described (*8*,*9*). The isolates from India had a distinct banding pattern in relation to isolates from Japan and South Korea. Except for 2 isolates (CBS12772, CBS12776), the *C. auris* isolates showed an identical banding pattern, suggesting a single genotype (Figure 1). The isolates' genotypic diversity was also determined by using amplified fragment length polymorphism (AFLP) fingerprint analysis (*10*). In a dendrogram in which standard Pearson and UPGMA (unweighted pair group method with averages) settings were used, *C. auris* isolates clustered separately from *C. haemulonii*, *C. duobushaemulonii*, and *C. pseudohaemulonii*. The *C. auris* isolates from India showed a clonal origin and were genotypically distinct from the isolates from Japan and South Korea (Figure 2).

Antifungal susceptibility testing was done by using microbroth dilution according to Clinical Laboratory Standards Institute protocols (*11*). All isolates showed high geometric mean MICs of fluconazole (Pfizer, Groton, CT, USA) (28.5 mg/L), whereas isavuconazole (Basilea Pharmaceutica, Basel, Switzerland [now Astellas]), posaconazole (Schering-Plough, Kenilworth, NJ, USA [now Merck]), itraconazole (Lee Pharma, Hyderabad, India), and voriconazole (Pfizer) exhibited highly potent activity.

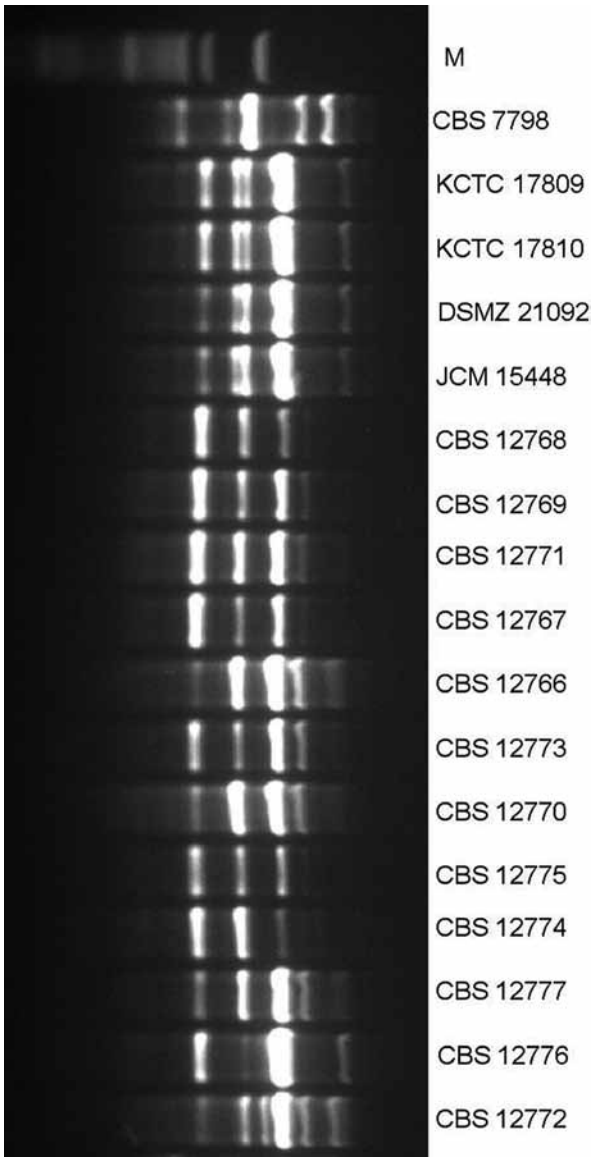

Figure 1. M13 PCR fingerprinting of *Candida auris* isolates. Lane 1, 50-bp ladder (New England BioLabs, Evry, France); lane 2, *C. duobushaemulonii* reference isolate (CBS7798); lanes 3–6, *C. auris* isolates from South Korea (KCTC 17809 and KCTC 17810), Japan (DSMZ21092 and JCM 15448), and reference isolates; and lane 7–18, 12 test isolates from India.

Amphotericin B (Sigma-Aldrich, St. Louis, MO, USA), flucytosine (Sigma-Aldrich), and the 3 echinocandins also showed excellent activity (Table).

Clinical features of the infections and response to antifungal therapy of the 12 patients with fungemia are summarized in the online Technical Appendix Table (wwwnc.cdc.gov/EID/article/19/10/13-0393-Techapp1.pdf). Each patient had at least 3 predisposing risk factors. Seven had immunosuppressive conditions: 6 (50%) had diabetes mellitus, 5 (42%) had chronic kidney disease, 2 (17%) had undergone cancer chemotherapy, and 1 (8%) was infected with HIV. Of the 5 children, 3 were infants of low birthweight, 1 neonate had late onset sepsis, and one 10-year-old child had acute lymphoblastic leukemia. Having an indwelling urinary catheter was the predominant risk factor for 83% of patients, followed by receiving broad-spectrum antimicrobial drugs (75%), spending time in an intensive care unit (58%), having neutropenia (50%), and having a central venous catheter (CVC) (42%). Notably, 7 (58%) patients had breakthrough fungemia while receiving fluconazole (1–3 weeks), and persistent candidemia developed in 9 (75%) patients. In 8 of these 9 patients, candidemia cleared after antifungal therapy in 1–3 weeks. For the remaining 3 patients, blood culture reports were available post mortem. *C. auris* represented 5% of the annual candidemia cases in a pediatric hospital and 30% of annual candidemia cases in a tertiary care general hospital. Overall mortality rates as high as 50% were noted; 30% of deaths were attributed to critical illness.

Conclusions

This study documents the emergence of a new clonal strain of *C. auris* as an etiologic agent of candidemia in India. Fungemia caused by *C. auris* is extremely rare, identified only by sequencing. Because diagnostic laboratories do not undertake molecular identification routinely, fungemia is likely to be much more prevalent than published reports indicate. Growth at 40°C may differentiate *C. auris* isolates, routinely misidentified as *C. haemulonii* by VITEK. *C. haemulonii* does not grow at 40°C, as does *C. auris* (*12*). Sequencing may confirm their identity.

Although Lee et al. proposed negative assimilation of NAG for differentiating *C. auris* from *C. pseudohaemulonii*, this proposal seems invalid because all isolates from India assimilated NAG (*4*). The phenotypic divergence of these isolates was also supported by M13 and AFLP typing. Notably, *C. auris* isolates from 2 hospitals in Delhi were clonal, suggesting interhospital transmission. Transmission of the same genotype was observed in intensive care, surgical, medical, oncologic, neonatal, and pediatric wards, which were mutually exclusive with respect to health care personnel. This finding is analogous to that of the clonal transmission of *C. auris* among 3 hospitals in South Korea (*2*). Prevalence of a unique clonal strain of *C. auris* in 2 hospitals in Delhi, 13.5 km apart, underscores the need for more comprehensive studies to determine potential occurrence of other endemic clones in various regions of India.

Because most (83%) of the patients in our study had an indwelling urinary catheter, the source of fungemia could be urogenital colonization. However, no urine samples were cultured. Five (42%) patients had a CVC in situ, but

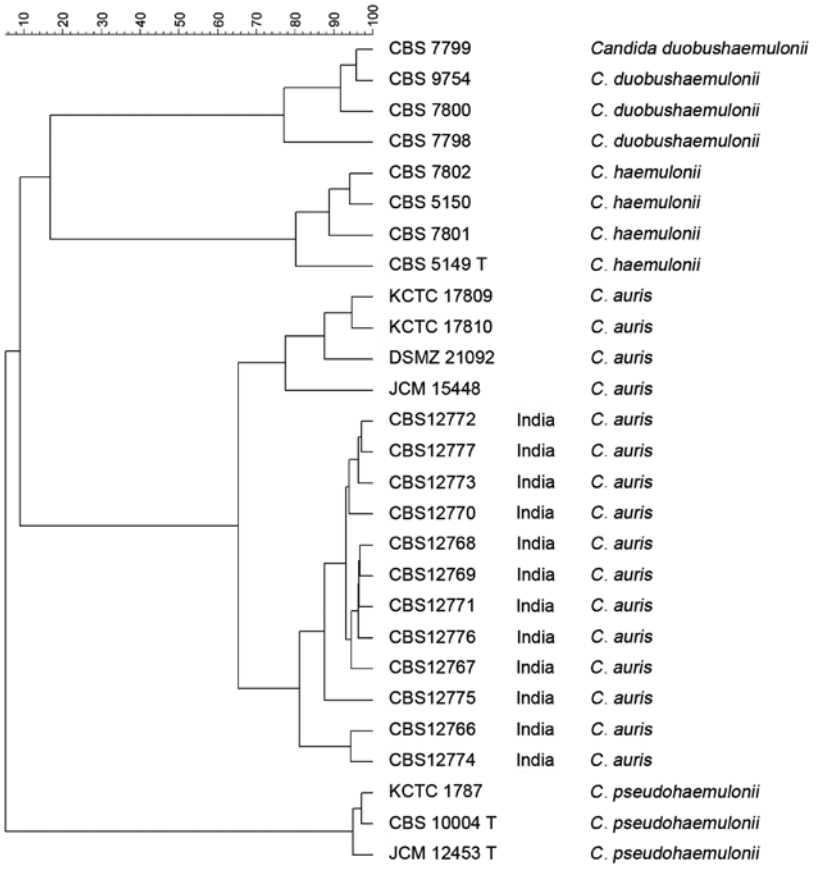

Figure 2. Dendrogram of amplified fragment length polymorphism analysis of *Candida auris* isolates from India, Japan, and South Korea and members of *C. haemulonii* complex. It was constructed by using UPGMA (unweighted pair group method with averages) in combination with the Pearson correlation coefficient and was restricted to fragments of 60–400 bp. Scale bar indicates the percentage similarity.

none received total parenteral nutrition. Although *Candida* spp. adhere to intravascular catheters, providing a potential nidus for infection, a previous study showed that *C. auris* does not form a biofilm (*3*). Catheter tip cultures were negative for 3 of the 5 patients who had a CVC. Although 57% of patients admitted to critical care units died, the role of *C. auris* candidemia in fatal cases could not be ascertained because no comparison was available with a population that did not have candidemia.

C. auris assumes a greater clinical significance for the successful management of candidemia because all the reported isolates, including the new isolates, were fluconazole resistant (*2*,*4*). Most patients in this study had persistent candidemia, and mortality rates were as high as 33%.

In conclusion, *C. auris* is an emerging yeast pathogen that is underreported because it is misidentified in routine diagnostic laboratories. The resistance of *C. auris* to fluconazole is particularly worrisome.

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Table. Results of In vitro antifungal susceptibility testing of 12 *Candida auris* isolates originating from fungemia patients in 2 hospitals, Delhi, India, 2009–2011

	Drug†									
MIC, mg/L*	AMB	FLU	ITC	VRC	ISA	POS	FC	CAS	MFG	AFG
MIC_{50}	0.25	32	0.125	0.5	0.125	0.125	0.125	0.25	0.06	0.25
MIC_{90}	0.5	64	0.25	1	0.25	0.25	0.125	0.25	0.125	0.5
GM	0.33	28.5	0.15	0.39	0.10	0.12	0.125	0.23	0.072	0.26
Range	0.25–1	16–64	0.125–0.25	0.125–1	<0.015–0.25	0.06–0.25	0.125	0.125–0.25	0.06–0.125	0.125–0.5

*MIC_{50}, 50% minimum inhibitory concentration; MIC_{90}, 90% minimum inhibitory concentration; GM, geometric mean of MICs.
†AMB, amphotericin B; FLU, fluconazole; ITC, itraconazole; VRC, voriconazole; ISA, isavuconazole; POS, posaconazole; FC, flucytosine; CAS, caspofungin; MFG, micafungin; AFG, anidulafungin.

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Subclinical Avian Influenza A(H5N1) Virus Infection in Human, Vietnam

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Laboratory-confirmed cases of subclinical infection with avian influenza A(H5N1) virus in humans are rare, and the true number of these cases is unknown. We describe the identification of a laboratory-confirmed subclinical case in a woman during an influenza A(H5N1) contact investigation in northern Vietnam.

In 2012, a debate was published in Science about the number of humans who have experienced subclinical infection with avian influenza A H5 and how this unknown denominator could affect the case-fatality rate reported by the World Health Organization (*1*,*2*). The controversy rests, to a large extent, on interpretation of serologic tests used to detect prior H5 infection and the paucity of virologically confirmed subclinical or mild cases. Here we describe a case of subclinical avian influenza A H5 infection, confirmed both virologically and serologically.

The Case

The subclinical case was detected in 2011 during a contact investigation of a 40-year-old man suspected of having influenza A(H5N1) virus infection. The man's household had sick poultry that were consumed by household members. The chickens roamed close to the sleeping area of the household members. The index case-patient, his daughter, and his daughter-in-law were involved in slaughtering and preparing the chickens. The index case-patient had fever, cough, dyspnea, and diarrhea that progressed over 2 days, leading to hospital admission. Despite intensive care and treatment with oseltamivir and antibiotics, the disease progressed, and he died 2 days later.

A throat swab taken from the index case-patient on day 3 of illness was tested by reverse transcription PCR, and results were positive for influenza A(H5N1) virus. Hemagglutination inhibition (HI) and microneutralization (MN) tests for H5N1-specific antibodies were negative in samples taken during the acute phase of illness (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0730-Techapp1.pdf).

On day 5 of illness of the index case-patient, a contact investigation was initiated. Throat swab specimens were collected from 4 household members and 1 close contact of the index case-patient: his spouse (age 47 years), daughter (age 18 years), daughter-in-law (age 25 years), and grandson (age 1 year) and an unrelated man (age 43 years). None of the contacts had signs or symptoms. Infection control measures were initiated, and all household members were given oseltamivir (75 mg/d) for 1 week and instructed to seek immediate health care if fever or respiratory symptoms developed. Results of HI testing of serum samples collected during the acute illness phase of the index case-patient were negative.

The human throat swab samples were tested by conventional RT-PCR. The sample from the index case-patient's daughter, collected 6 days after the woman had slaughtered a chicken, was positive for influenza A/H5 by real-time RT-PCR, and virus was recovered on day 10 of inoculation on MDCK cells (online Technical Appendix). The virus was identified by sequencing as influenza A/H5, clade 2.3.2.1. The woman had no signs or symptoms at the time the throat swab was collected, nor did she report any symptoms to health authorities during the subsequent week. Chickens were also tested, and 4 chickens in the commune tested positive for influenza A(H5N1) virus by RT-PCR of throat and cloacal swab specimens.

Repeat throat swab specimens collected from the 4 household contacts 6 days after the initial collection yielded negative test results for influenza/H5. Serologic testing showed seroconversion only in the woman with subclinical infection; her HI titer increased from <20 to 160 against both clade 2.3.4 and 2.3.2.1 viruses (Table 1). During a second contact investigation 1 month later, 20 other members of the commune were screened by RT-PCR of throat swab specimens and serologic testing. All results were negative for influenza A(H5N1) virus.

The full genome of the identified virus strain (A/CM32/2011) was sequenced (online Technical Appendix) and confirmed to be clade 2.3.2.1 by using the Highly Pathogenic H5N1 Clade Classification Tool (*3*). The open reading frames of the genes were translated and aligned with all clade 2.3.2.1 sequences available from

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Table 1. HHI and MN assay titers for serum samples from woman with subclinical influenza A(H5N1) virus infection, Vietnam, 2011

Clade and sample	HHI	MN
Clade 1		
First sample, day 5	<20	ND
Second sample, day 11	40	<10
Third sample, day 41	40	<10
Clade 2.3.2.1		
First sample, day 5	<20	ND
Second sample, day 11	80	80
Third sample, day 41	160	160
Clade 2.3.4		
First sample, day 5	<20	ND
Second sample, day 11	80	40
Third sample, day 41	160	40

*Sample days are days since disease onset in index case-patient. HHI, horse hemagglutination inhibition; MN microneutralization; ND, not done.

the Influenza Research Database (www.fludb.org). Amino acid changes are summarized in Table 2 (Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0730-T2.htm). A phylogenetic analysis of clade 2.3.2.1 from Vietnam sequences showed a high homology between the samples, including A/CM32/2011 (Figure). To identify possible changes specific to human infection, the differences between the clade consensus and A/CM32/2011 were compared with influenza A(H5N1) virus samples from Asia. No amino acid changes were preferentially seen in human samples compared with the avian samples. A/CM32/2011 contains the N170D (*4*) and T172A mutations in HA that are associated with airborne transmissibility of influenza A(H5N1) virus in ferrets (*5*); these mutations are found in most avian influenza A(H5N1) clade 2.3.2.1 samples (Table 2, Appendix). The results indicate that the virus is a typical influenza A(H5N1) clade 2.3.2.1 virus, with no remarkable changes.

Conclusions

We report subclinical infection with avian influenza A(H5N1) virus in a human in Vietnam, confirmed by RT-PCR, virus isolation from throat swab, and detection of specific antibodies. A subclinical case was also reported from Pakistan in 2008 (*6*). Sequence analysis of the Vietnam case showed that the infecting virus belonged to influenza A(H5N1) clade 2.3.2.1. This clade was first detected in poultry in northern Vietnam in early 2010 and replaced clade 2.3.4 in that area, whereas clade 1 remains predominant in southern Vietnam, with 4 confirmed cases reported in early 2012 (*7*). The recent clade 2.3.2.1 has evolved from clade 2.3.2 viruses that has circulated among poultry in eastern Asia since 2005 and has become predominant in several Asian countries. Since clade 2.3.2.1 viruses were initially detected in Vietnam, prevalence has increased in poultry, but no associated rise in detection of human cases has been observed (*7*). Similarly, clade 2.3.2.1 virus has been circulating in poultry in India, but no human cases have been reported (*8*). The HA sequence of this virus is similar to an influenza A(H5N1) virus detected by RT-PCR in a 3-year-old patient with influenza-like illness investigated as part of the National Influenza System Surveillance in 2010 (M.Q. Le, unpub. data). This patient had mild symptoms and survived, which raises the possibility that this strain represents a less virulent form of influenza A(H5N1) in humans.

In our investigation, the case-patient with subclinical infection was treated with oseltamivir while she was asymptomatic, which may explain why she did not develop clinical disease. Studies using human volunteers indicate that seasonal influenza virus shedding may occur ≈24 hours before symptom onset in 25%–30% of patients (*9*). Likewise, community cohort studies show presymptomatic shedding and asymptomatic shedding in 15%–20% of patients infected with seasonal influenza viruses and with influenza A(H1N1)pdm09 virus (*10*–*12*). Oseltamivir is known to prevent disease when given before inoculation in human volunteers and to shorten duration and lessen the severity of illness in natural infection (*13*), but we found no evidence in clinical and volunteer studies from the literature suggesting that oseltamivir may prevent clinical illness once detectable infection has been established, as we found in this subclinical case (*13*,*14*). The patient we investigated probably was exposed during slaughtering of a chicken 6 days before her positive throat swab was collected. However, because chickens in the commune tested positive at the time of the contact investigation, ongoing exposure to influenza A(H5N1) cannot be excluded as the source of infection. Furthermore, the patient may not have reported symptoms to the health authorities for personal reasons.

Thus far, evidence of subclinical influenza A(H5N1) virus infections has been collected on the basis of serologic testing only (*1*), but it is unclear whether serologic testing reliably detects subclinical cases. According to the World Health Organization, MN titers ≥80 are indicative of infection but must be confirmed by a second serologic test because of the possibility of cross-reactivity (*1*). The interpretation of results from a single serum sample is limited by the specificity or sensitivity of serologic tests, and viral shedding times may mean that infected cases may be missed. Estimating the incidence of asymptomatic influenza A(H5N1) virus infection in humans exposed to sick poultry or human case-patients requires further careful study using early collection of swab samples and paired acute and convalescent serum samples.

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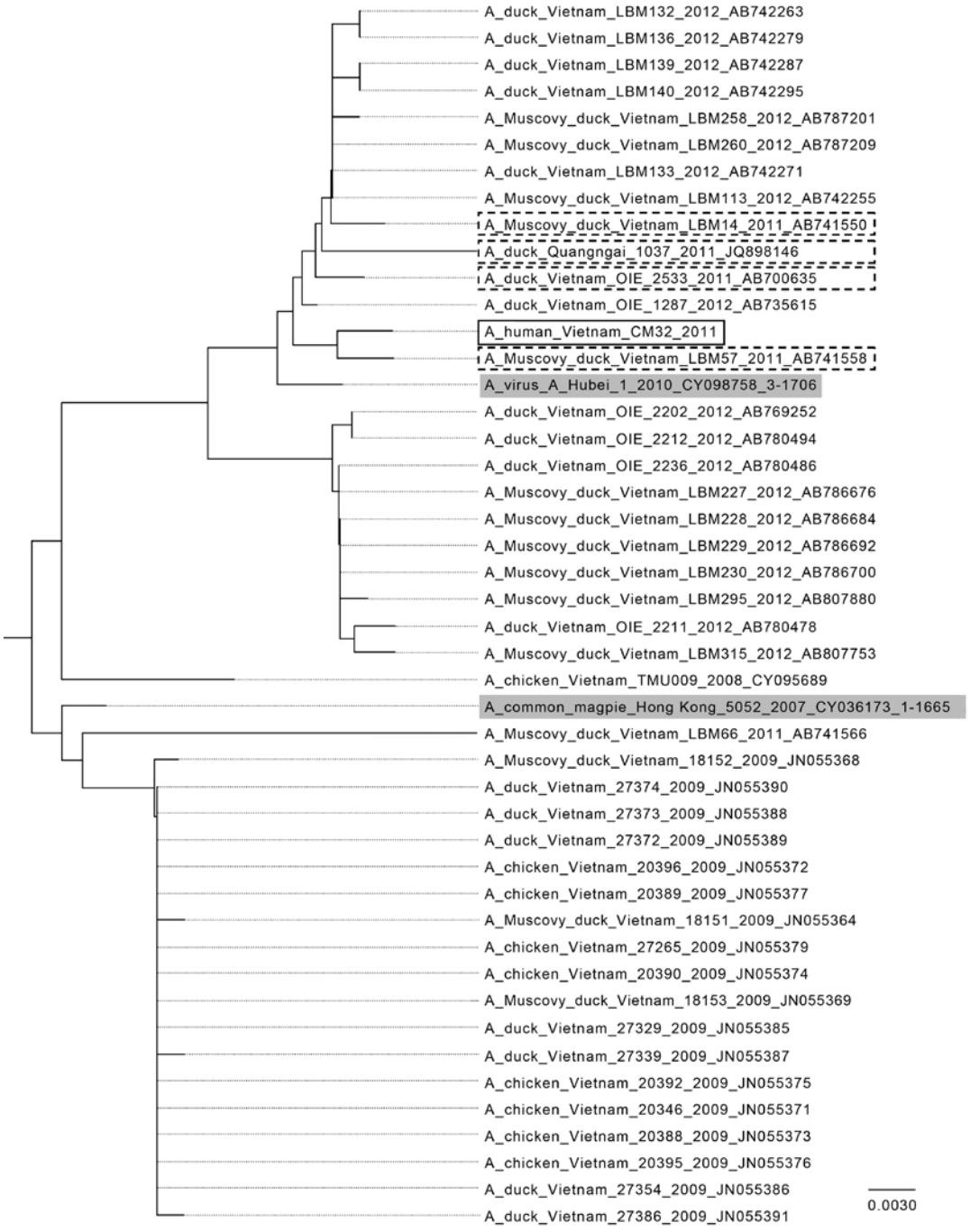

Figure. Phylogentic analysis of avian influenza A(H5N1) virus clade 2.3.2.1 hemagglutinin DNA sequences from Vietnam compared with other isolates. Solid black box indicates isolate from the subclinical human case investigated in this study, A/CM32/2011; dashed boxes indicate sequences from Vietnam in 2011; gray shading indicates World Health Organization vaccine candidates A/common magpie/Hong Kong/5052/2007 and A/Hubei/1/2010 for clade 2.3.2.1. The sequences were downloaded from the Influenza Research Database (www.fludb.org), imported into MEGA 5.2 (www.megasoftware.net), and aligned by using MUSCLE (EMBL-EBI, Cambridgeshire, UK). The neighbor-joining tree was generated from the aligned sequences using standard settings. Scale bar indicates nucleotide substitutions per site.

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Human Infections with New Subspecies of *Campylobacter fetus*

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Campylobacter fetus subsp. *testudinum* subsp. nov. is a newly proposed subspecies of *C. fetus* with markers of reptile origin. We summarize epidemiologic information for 9 humans infected with this bacterium. All cases were in men, most of whom were of Asian origin. Infection might have been related to exposure to Asian foods or reptiles.

Campylobacter spp. are the most common cause of bacterial gastrointestinal illness in humans. *C. jejuni* is the most common species and accounts for >88% of reported cases in the United States (*1*). *C. fetus* is an uncommonly reported species that typically affects immunocompromised, pregnant, or elderly persons and causes severe infections, including bacteremia and meningitis (*2*).

Two subspecies of *C. fetus* have been described: *C. fetus* subsp. *fetus* and *C. fetus* subsp. *venerealis* (*3*). *C. fetus* subsp. *fetus* has been isolated from intestinal tracts of sheep and cattle and from tissues from sporadic abortions in these species. *C. fetus* subsp. *venerealis* is restricted to cattle and causes bovine genital campylobacteriosis (*4*). Although *C. fetus* subsp. *venerealis* has been isolated from humans (*5*), its role in human disease is uncertain; most cases of *C. fetus* infection are caused by *C. fetus* subsp. *fetus*.

In 1984, *C. fetus* was isolated from feces of a reptile, a Florida box turtle (*Terrapene carolina bauri*) that was kept as a pet (*6*). *C. fetus* has also been isolated from feces of a healthy western hognose snake (*Heterodon nasicus*) and a blotched blue-tongue lizard (*Tiliqua nigrolutea*) that had unformed feces and was losing weight (*7*). Substantial genetic divergence between *C. fetus* strains of reptile and mammal origin has been demonstrated (*8*).

A human isolate of *C. fetus* with markers of reptile origin was reported in 2004 (*9*). A subsequent study involving phenotypic and molecular characterization of the 2004 human case, 4 additional human cases, and 3 reptiles definitively identified this collection of strains as a newly proposed subspecies named *C. fetus* subsp. *testudinum* subsp. nov. (*7,10*). The Centers for Disease Control and Prevention recently screened *Campylobacter* strains from its historical culture collection and identified 4 additional human cases of infection with this subspecies.

The Study

We collected demographic and epidemiologic information to describe characteristics of the 9 reported patients infected with *C. fetus* subsp. *testudinum* subsp. nov. Food preferences and limited information about exposures were available for 5 patients. Four patients could not be interviewed because they could not be located or had died. However, some information was available from their original case reports. We summarize our findings in the Table.

Patients resided in Colorado, Louisiana, Massachusetts, and New York, and had onset of illness during 1991–2010. All patients were men (median age 73 years, range 20–85 years). Five of 6 patients were of Asian origin (4 were Chinese and 1 was either Chinese or Vietnamese), and the non-Asian patient had a Chinese spouse. Last names of the remaining 3 patients did not suggest that they were of Asian origin.

C. fetus subsp. *testudinum* subsp. nov. was isolated from blood (4 patients), feces (2 patients) pleural fluid (1 patient), hematoma (1 patient), and bile (1 patient). Of 5 patients with available information, all had underlying illness. Clinical symptoms varied. One patient had fever, cough, and epigastric pain; another had fever, chills, rigors, cough, and diarrhea; and a third had bloody feces, pulmonary edema, and pleural effusion. One patient sought care for dizziness and mental confusion after a fall, and *C. fetus* subsp. *testudinum* subsp. nov. was isolated from a subdural hematoma. For another patient, *C. fetus* subsp. *testudinum* subsp. nov. was isolated from blood after cellulitis developed from a leg wound; no gastrointestinal symptoms were reported. All 6 patients for whom outcomes were available were hospitalized, and 1 died of leukemia.

All 5 patients of Asian origin and the 1 patient with an Asian spouse shopped or ate at restaurants in Chinese (Chinatown) areas in Massachusetts and New York. A limited food and travel history was available for 4 patients. All 4 reported eating traditional Chinese dishes. In addition, 1 patient ate eel, 1 ate eel and frog, 1 ate turtle soup, and 1

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Table. Characteristics of 9 men with *Campylobacter fetus* subsp. *testudinum* subsp. nov. infection, 1991–2010*

Pt no.	Pt age, y	Year	Ancestry	Isolate/ strain no.	Specimen source	Clinical symptoms	Underlying conditions	Hospitalized	Died	Food history	Animal contact
1	NA	1991	NA	D6783/91-2	Feces	NA	NA	NA	No	NA	NA
2	30	1992	NA	D4335	Feces	Diarrhea	NA	NA	NA	NA	Turtle
3	80	2002	NA	D6128	Blood	NA	NA	NA	NA	NA	NA
4	20	2003	Chinese	D6688/ 03-0427, D6689/ 03-445†	Blood	Fever, cough, epigastric pain	Leukemia, hepatitis B	Yes	Yes	Turtle soup, angelica herb	NA
5	79	2004	Chinese or Vietnamese	D6659	Pleural fluid	Bloody diarrhea, pulmonary edema	Liver cancer	Yes	NA	NA	No
6	84	2005	Chinese	D6683	Hematoma	Speech and motor difficulties, hematoma	Asthma	Yes	No	Chinese dishes, eel, frog	No
7	67	2005	Chinese	D6690/ 05-018	Blood	Fever, chills, rigor, cough, diarrhea	Lymphoma, hypertension, heart disease	Yes	No	Chinese dishes	No
8	85	2007	Chinese	D6856	Bile	Diarrhea	NA	Yes	NA	NA	No
9	62	2010	Caucasian‡	D9240	Blood	Cellulitis of leg	Diabetes	Yes	No	Chinese dishes, eel	No

*Pt, patient; NA, not available.
†Two isolates were obtained from this patient.
‡Asian spouse.

denied eating turtle or frog. Three patients did not report any recent travel, and 1 reported frequent travel, including trips to Europe and Hong Kong. Food and travel histories were not available for the non-Asian patients. However, 1 patient reported contact with a turtle that had diarrhea. This patient did not appear to have had a systemic infection; his isolate was obtained from feces, and he reported a 16-day history of diarrhea.

Conclusions

C. fetus subsp. *testudinum* subsp. nov. is a newly proposed subspecies that appears to have originated in reptile species. Although information is limited, our data suggest that humans may contract this subspecies though exposure to reptiles, possibly by ingestion or by contact with feces or the environment. Reptiles, particularly small turtles, are a well-known source of *Salmonella* spp. infections in humans (*11*). A recent study in Taiwan reported *C. fetus* subsp. *testudinum* subsp. nov. in feces of 12 (6.7%) of 179 reptile feces samples; prevalence was highest in turtles (10 [9.7%] of 103) (*12*). Turtle is an ingredient in some traditional Asian dishes and turtles are frequently sold in Asian specialty markets; this association might partly explain the predominance of Asian race among reported patients. A review of GenBank found 5 submissions of 16S rDNA sequences representing *C. fetus* subsp. *testudinum* subsp. nov. from China (accession nos. DQ997044, HQ450384, HQ681195, JN585921, and JN585922).

Although *C. fetus* is more common among men (*13,14*), it is unusual that all of the recognized patients infected with *C. fetus* subsp. *testudinum* subsp. nov. were men. This finding might indicate that men have a predisposition to infection with this subspecies or that they are more likely to be exposed to sources of contamination. Nearly all patients with *C. fetus* subsp. *testudinum* subsp. nov. infections were >60 years of age or had immunocompromising conditions. This finding indicates that *C. fetus* subsp. *testudinum* subsp. nov., like *C. fetus* subsp. *fetus*, are opportunistic pathogens that might lead to severe disease. Most patients had primary bacteremia; only 4 of 7 patients had diarrhea associated with their illnesses.

The actual number of *C. fetus* subsp. *testudinum* subsp. nov. illnesses is unknown. *C. fetus* infections are likely to be underdiagnosed and underreported. *C. fetus* is susceptible to cephalosporins, which are commonly included in media used for isolation of *Campylobacter* spp., making *C. fetus* isolation from feces unlikely. *Campylobacter* spp. infection is not a nationally reportable disease in the United States, and in most states, isolates are not routinely sent to state public health laboratories for confirmation of identification. Although some clinical and state laboratories identify *Campylobacter* spp., this identification is not conducted routinely, and few laboratories use molecular methods to identify strains to the species level. For *C. fetus* subsp. *testudinum* subsp. nov., additional identification methods have to be performed. Therefore, we encourage laboratories that identify the *Campylobacter* spp. to forward isolates of *C. fetus* to the *Campylobacter* Reference Laboratory at the Centers for Disease Control and Prevention for confirmation and screening for *C. fetus* subsp. *testudinum* subsp. nov. In addition, when interviewing persons with *Campylobacter* spp. infections,

public health personnel should ask about exposure to live reptiles and traditional Asian dishes made with turtles or other reptiles.

In summary, our data show that *C. fetus* subsp. *testudinum* subsp. nov. can cause invasive infection. All known cases have occurred in men, most of whom were of Asian origin, and infection may be related to exposure to traditional Asian foods or reptiles. Persons who are immunocompromised should avoid eating undercooked reptiles, exposure to live reptiles, and their environments. Enhanced public health surveillance and laboratory testing and surveys of the prevalence of the organism in reptiles are needed to better understand the epidemiology and incidence of *C. fetus* subsp. *testudinum* subsp. nov. and to recommend additional prevention measures.

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Chikungunya Fever Outbreak, Bhutan, 2012

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In 2012, chikungunya virus (CHIKV) was reported for the first time in Bhutan. IgM ELISA results were positive for 36/210 patient samples; PCR was positive for 32/81. Phylogenetic analyses confirmed that Bhutan CHIKV belongs to the East/Central/South African genotype. Appropriate responses to future outbreaks require a system of surveillance and improved laboratory capacity.

Chikungunya fever is caused by infection with chikungunya virus (CHIKV; family *Togaviridae*, genus *Alphavirus*). CHIKV was first isolated from humans and mosquitoes during an epidemic of chikungunya fever in Newala, Tanzania, during 1952–1953 (*1*). The virus is transmitted to humans primarily by *Aedes aegypti* and *Ae. albopictus* mosquitoes. Chikungunya fever can cause lingering joint pain, often lasting several weeks; the disease is rarely life-threatening and, unlike dengue virus infection, does not cause severe hemorrhagic manifestations or shock (*2*). Outbreaks of chikungunya fever are sporadic, and there is no licensed vaccine to protect against the disease.

In addition to the chikungunya fever epidemic in Tanzania in 1952–1953, several other outbreaks have affected millions of persons in eastern, western, and central Africa and Asia. The first of many chikungunya fever outbreaks in Southeast Asia was reported in Bangkok, Thailand, in 1958, followed by outbreaks in Sri Lanka; Kolkata and Chennai, India; and elsewhere (*3*–*6*). CHIKV reemergence in Indonesia and India caused epidemics during 1999–2003 and 2005–2006, respectively; 1.3 million cases of chikungunya fever occurred in India during the epidemics (*6*,*7*). In 2004, a chikungunya fever outbreak occurred in the Indian Ocean region. On Réunion Island, 266,000 cases, affecting ≈40% of the population, and ≈250 deaths were recorded (*7*). We report details of a chikungunya fever outbreak in Bhutan in July 2012; CHIKV had not been reported in Bhutan before this outbreak.

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The Study

In July 2012, the Public Health Laboratory (PHL), Department of Public Health, Ministry of Health, in Thimphu, Bhutan, received reports of suspected chikungunya fever cases. A total of 215 acute-phase serum samples were obtained from patients at hospitals in the southwestern districts of Samtse, Chukha, and Thimphu (online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/10/13-0453-Techapp1.pdf) and sent to PHL for testing (Table 1) (*8*). Samples were maintained at 4°C until arrival at PHL. Most patients from whom a serum sample was obtained were inpatients 15–44 years of age. Of the 215 samples, 84 (39%) were from female patients (mean age of 27.2 years, range 4 months to 68 years, and 131 (61%) were from male patients (mean age 31.5 years, range 2–78 years).

Serum samples from 210 of the 215 patients were tested for CHIKV IgM by ELISA (Table 1). The CHIKV ELISA was developed by the National Institute of Virology in Pune, India, and supplied to PHL by the World Health Organization. Of the 210 samples, 36 (17.1%) were positive for CHIKV IgM. As determined by Fisher exact test, there were no significant differences in the percentage of IgM ELISA–positive cases in the 3 districts ($p \geq 0.2$) or for male versus female patients ($p \geq 0.08$). Medical records were provided for only 79 patients; significantly fewer records were provided for patients in Chuka than for patients in Samtse and Thimphu ($p \geq 0.003$). Of the 79 patients with medical records, all had a fever, 48 (60.1%) had arthralgia, 55 (69.6%) had headache, and 6 (7.6%) had a rash (*8*). There were no substantial differences in the distribution of symptoms by patient sex of district of residence.

The first 78 (37%) acute-phase serum samples received by PHL were shipped frozen to the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, for testing by nested reverse transcription PCR (RT-PCR) amplification of the capsid gene (Table 2) (*9*). Of these 78 samples, 32 (41%) were positive for CHIKV. The difference in distribution of positive and negative RT-PCR results by patient sex was not statistically significant in any of the 3 districts ($p \geq 0.1$, Fisher exact test). Of 8 IgM ELISA–positive samples sent to AFRIMS for testing, 4 (50%) were positive by RT-PCR, and of 67 IgM ELISA–negative samples sent to AFRIMS for testing, 27 (40.3%) were positive (Table 2).

The first RT-PCR–confirmed CHIKV-positive specimen was collected on July 12, 2012, from a patient for whom the date of illness onset was listed as July 11. On July 9, the patient had traveled through West Bengal, India, en route from Thimphu to Samtse, Bhutan. All confirmed chikungunya fever case-patients in Thimphu had traveled to Phuentsholing, Bhutan, or other places in southern Bhutan affected by the outbreak.

Table 1. IgM ELISA results and signs and symptoms for patients with suspected chikungunya fever, Bhutan, 2012

District, patient sex	No. patients	Mean age, y	No. positive samples/no. tested (%)	No. patients for whom medical history obtained	No. (%) patients with sign/symptom			
					Fever	Arthralgia	Headache	Rash
Chuka								
F	21	19.6	2/21 (9.5)	3	3 (100)	1 (33)	0	0
M	28	28.8	8/27 (29.6)	5	5 (100)	2 (40)	3 (60)	0
Samtse								
F	40	28.2	3/39 (7.7)	25	25 (100)	14 (56)	19 (76)	3 (12)
M	50	28.2	7/47 (14.9)	30	30 (100)	16 (53)	20 (67)	1 (3)
Thimphu								
F	23	32.4	6/23 (26.1)	2	2 (100)	2 (100)	2 (100)	0
M	53	36.1	10/53 (18.9)	14	14 (100)	13 (93)	11 (79)	2 (14)
Total	215	–	36/210 (17)	79	79 (100)	48 (60.1)	55 (69.6)	6 (7.6)

Six specimens, which were selected on the basis of their relatively strong CHIKV RT-PCR results, were used for partial sequencing of the envelope protein 1 (E1) gene; all 6 specimens were from Samtse. The 1,320-bp sequences were compared against 34 other GenBank E1 sequences from different localities (Figure 1). The 6 representative isolates (GenBank accession nos. KC731581–KC731586) were 99.9%–100% identical at the nucleotide level and 99.8% identical at the amino acid level. Phylogenetic analyses of the partial E1 sequences showed that the 2012 outbreak of chikungunya fever in Bhutan was caused by a CHIKV of the East/Central/South African genotype, and the virus was removed only by 0.5% from HM159388 India 2010 CHIKV, 0.7% from GQ229488 India 2009 CHIKV, and 0.5% from HM045801 Sri Lanka 2007 CHIKV. Amino acid sequence analyses showed that all 6 Bhutan isolates lack the E1 gene mutations, which led to the A226V E1 protein change found in the Réunion Island 06.49 strain and which are closely associated with a higher transmission rate (Figure 2) (*10*). In addition, the 2012 Bhutan strain still retained the E211 amino acid, which is also found in the India 2010 strain (*11*).

Conclusions

The results of our phylogenetic analyses suggest that the Bhutan CHIKV originated from CHIKV found in India. This finding is not surprising given the proximity of the 2 countries and the expanding range of the virus. The 2012 chikungunya fever outbreak in Bhutan occurred in the same area as a 2004 outbreak of dengue virus infection (*12*); this area shares borders with West Bengal and Assam, India.

The IgM ELISA and RT-PCR results in our study did not always agree; however, the discrepancies may be explained by the time of collection relative to onset of symptoms. Specimens collected early after illness onset were likely collected during the viremic state and thus positive by RT-PCR, but not by IgM ELISA. Specimens collected later after illness onset would likely have been IgM positive, even in the absence of detectable virus.

Table 2. IgM ELISA and PCR results for 78 patients with suspected chikungunya fever, Bhutan, 2012*

District, patient sex, no. patients	IgM ELISA†			Nested reverse transcription PCR‡	
	No. positive/no. tested (%)	No. negative/no. tested (%)	No. not tested/no. total (%)	No. positive/no. tested (%)	No. negative/no. tested (%)
Chuka§					
F, n = 6	0/6			NA	NA
		6/6 (100)		2/6 (33)	4/6 (67)
M, n = 10	0/10			NA	NA
		10/10 (100)		3/10 (30)	7/10 (70)
Samtse¶					
F, n = 25	1/24 (4)			0/1	1/1 (100)
		23/24 (96)		12/23 (52)	11/23 (48)
			1/25 (4)	1/1 (100)	0/1
M, n = 34	6/32 (19)			4/6 (67)	2/6 (33)
		26/32 (81)		9/26 (35)	17/26 (65)
			2/34 (6)	0/2	2/2 (100)
Thimphu‖					
F, n = 1	0/1			NA	NA
		1/1 (100)		0/1	1/1 (100)
M, n = 2	1/2 (50)			0/1	1/1 (100)
		1/2 (50)		1/1 (100)	0/1
Total	8/75 (11)	67/75 (89)	3/59 (5)	32/78 (41)	46/78 (59)

*NA, not applicable.
†Results reported by the Public Health Laboratory, Department of Public Health, Ministry of Health, Thimphu, Bhutan.
‡Results reported by the Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand.
§Mean age for female patients was 28.1 y; mean age for male patients was 26.5 y.
¶Mean age for female patients was 28.8 y; mean age for male patients was 28.7 y.
‖ Mean age for female patients was not reported; mean age for male patients was 31 y.

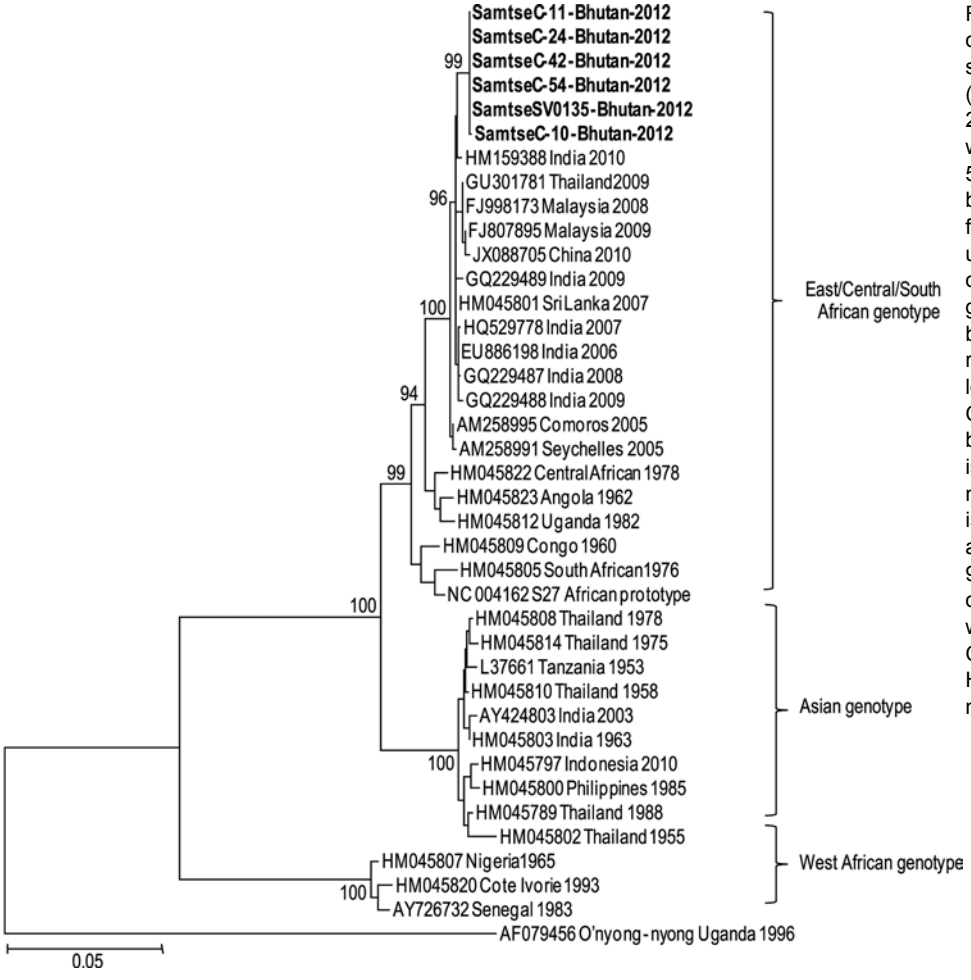

Figure 1. Phylogenetic analyses of the envelope protein 1 (E1) sequences of chikungunya virus (CHIKV) isolated in Bhutan in 2012. The neighbor-joining tree was constructed by using MEGA 5.0 (www.megasoftware.net); bootstrap values were obtained from 2,000 replicates. The unrooted tree topology was based on multiple alignments of the E1 gene nucleotide sequences (1,320 bp) and 34 CHIKV E1 regions, representing a wide range of localities, from GenBank. Each CHIKV isolate is represented by the country of origin, year of isolation, and GenBank accession number. Six representative isolates from the 2012 outbreak are included; the isolates show 99.9%–100% nt identity with each other and 99.4%–99.5% nt identity with circulating Indian 2012 CHIKV (GenBank accession no. HM159388). Scale bar indicates nucleotide substitutions per site.

Because of several factors, the introduction of CHIKV virus into Bhutan was expected: the presence of the mosquito vectors (*Ae. aegypti* and *Ae. albopictus*) in southern districts, the increasing geographic reach of CHIKV, the predominance of a presumably naive population, urbanization and industrial growth in Bhutan, and the country's unrestricted border with India. Another contributing factor may have been the circulation of a new East/Central/South African CHIKV strain, first isolated in 2005 from a patient on Réunion Island, that represents a distinct clade within the larger East/Central/South African phylogroup (*13*). This clade has the A226V mutation in the E1 protein, which leads to higher transmission and replication in *Ae. albopictus* mosquitoes (*10*). However, isolates collected during this outbreak lack this mutation and instead are more closely associated with the India 2010 strain (Figure 2).

The 2004 dengue virus infection outbreak (*12*) and now the 2012 chikungunya fever outbreak in Bhutan suggest that despite its remote location, the country is still

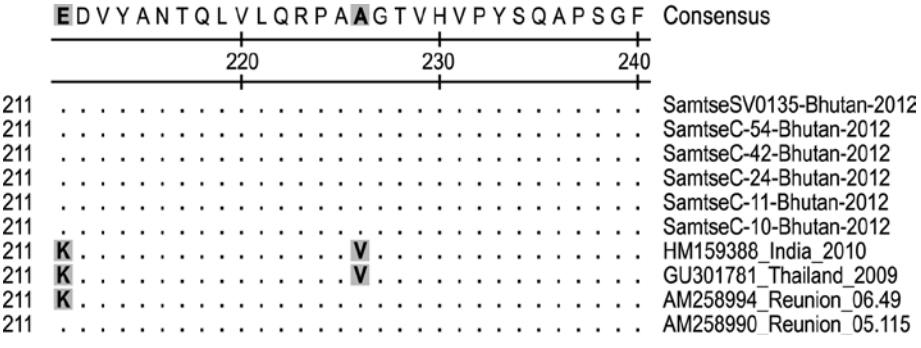

Figure 2. Amino acids at positions 211 and 226 of the chikungunya virus (CHIKV) envelope protein 1 (E1) and alignment of aa 211–240 of Bhutan 2012 CHIKV isolates with Réunion Island 2005, Indian 2010, and Thailand 2009 CHIKV isolates. Gray shading indicates residues 211 and 226 of the E1 protein.

vulnerable to epidemics and pandemics. A sound public health surveillance system and improved laboratory capacity is needed in Bhutan so that the country can appropriately respond to future disease outbreaks.

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Safe Pseudovirus-based Assay for Neutralization Antibodies against Influenza A(H7N9) Virus

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Serologic studies are urgently needed to assist in understanding an outbreak of influenza A(H7N9) virus. However, a biosafety level 3 laboratory is required for conventional serologic assays with live lethal virus. We describe a safe pseudovirus–based neutralization assay with preliminary assessment using subtype H7N9–infected samples and controls.

A novel reassortant avian influenza A(H7N9) virus has emerged in eastern China and caused fatal infections in humans (*1*–*3*). The real-time reverse-transcription PCR (RT-PCR) is used as a diagnostic method for detection of subtype H7N9 in infected patients or birds within the window of time when virus shedding is expected. Because of the pathogen-specific immune memory response in persons with asymptomatic cases or patients who have cleared infection, serologic assays are invaluable tools for estimating the incidence and prevalence in the population affected by the outbreak. However, those studies were confined to conventional hemagglutination-inhibition (HI) or microneutralization (MN) assays that are limited to propagation of the live lethal subtype H7N9 influenza virus in biosafety level 3 (BSL-3) laboratories. We describe a rapid and safe pseudovirus-based assay for detecting subtype H7N9 neutralizing antibodies. This assay can be performed in most laboratories equipped with BSL-2 facilities.

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The Study

Using a previously reported method (*4*), we assembled the pseudovirus with membrane proteins of hemagglutinin (HA) and neuraminidase (NA) from influenza A/Shanghai/4664T/2013(H7N9) and capsid protein from HIV. The genomic RNA of the pseudovirus also carries a luciferase reporter gene; thus its infectivity can be quantified by luciferase activity in virus-infected cells (*5*–*7*). To produce the pseudovirus, we carried out the following procedures: 4.5×10^6 293T cells cultured in a 10-cm dish were co-transfected with 5 μg of lentivirus vector pNL4-3-Luc R-E-, 2.5 μg of pVKD-HA, and 2.5 μg of pVKD-NA by using lipofectamine 2000 from Invitrogen (cat no. 11668; Carlsbad, CA, USA). The coding sequences of HA (GenBank accession no. KC853228) and NA (GenBank accession no. KC853231) were amplified from A/Shanghai/4664T/2013(H7N9) by using RT-PCR. (These constructs of pVKD-HA and pVKD-NA can be provided on request from the authors.) After overnight incubation, cells were washed once with phosphate-buffered saline and cultured in 5 mL complete Dulbecco minimum essential medium. The pseudovirus containing supernatant was harvested at 48 hours and stored at -80°C in aliquots until used in the neutralization assay. MDCK cells were infected with serially diluted pseudovirus stock, and the infectivity reflected by the relative luciferase activity (RLA) was determined as the median (50%) tissue culture infective dose, according to the method of Reed and Muench.

We then implemented the pseudovirus assay to measure neutralizing antibodies in clinical samples with known HI titers for subtype H7N9. Fourteen convalescent-phase serum samples with real-time RT-PCR–confirmed infection with 2013 subtype H7N9 were from patients 56–81 years of age from whom samples were collected 12–32 days after onset of symptoms and who were hospitalized in Shanghai Public Health Clinical Center; control samples were 50 stored serum samples collected in 2009 before the emergence of 2013 subtype H7N9. The neutralizing activities in patients' serum samples were also validated by the MN assay against live virus. In a 96-well plate, 2-fold serially diluted serum samples beginning at 1:10 were incubated with 200 median (50%) tissue culture infective doses of pseudovirus at the final volume of 100 μL at 37°C for 1 hour; then the mixture was added to the culture of MDCK cells. After incubation overnight, the cells were washed with 200 μL of phosphate-buffered saline and cultured in complete Dulbecco minimum essential medium for 48 hours in the original 96-well plate. RLA was measured by BrightGlo luciferase substrate (Promega, Madison, WI, USA; cat. no. E2610). Inhibition percentage was calculated as the following: (RLA in virus challenge controls – RLA

[1]These authors contributed equally to this article.

in test well for each serum at specific dilution)/RLA in virus challenge controls. The 50% inhibitory concentration (IC_{50}) titer was determined by the reciprocal of the last dilution that resulted in ≥50% reduction of luciferase activity. As expected, the IC_{50} titer quantified by the pseudovirus-based assay significantly correlated with HI titer measured by the inhibition of 1% guinea pig erythrocytes hemagglutination when incubated for 1 hour with reacted mixture of diluted serum samples and live virus (Figure; Spearman r = 0.88, p<0.0001, n = 64).

To determine the criteria delineating seropositive samples from seronegative samples for the pseudovirus-based assay, we tested the agreement between the HI assay and pseudovirus-based assay with the thresholds of 20 and 40, respectively. The strongest concordance between these assays was achieved by using 40 for the pseudovirus-based assay as the cutoff titer (Table; κ = 0.904; McNemar test, p = 0.5 for 1:40 and κ = 0.788; McNemar test, p = 0.375 for 1:20). With 40 as the cutoff, 12 (86%) of 14 HI assay samples positive for subtype H7N9 had an IC_{50} titer ≥40, whereas none of the control samples had an IC_{50} titer ≥40 (Table). Thus, in our preliminary assessment using this limited number of samples, the sensitivity of this assay was 85.7 (95% CI 0.572–0.982), and the specificity was 1.000 (95% CI 0.929–0.978). In addition, the serum samples used as negative controls contained antibodies against contemporary circulating seasonal influenza viruses, including H1 and H3 subtypes, which also supported the strain specificity of this subtype H7N9 neutralization assay (data not shown).

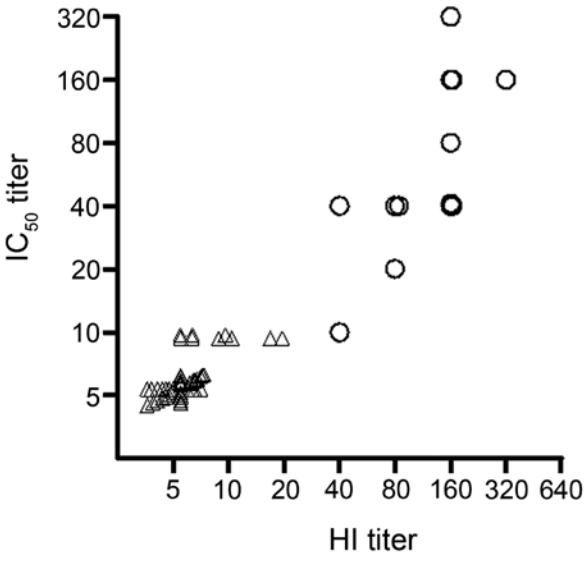

Figure. Correlation between conventional hemagluttination (HI) titer and 50% inhibitory concentration (IC_{50}) titer of pseudovirus-based assay for diagnosing influenza A(H7N9) virus infection. Correlation between the IC_{50} titer of the pseudovirus-based neutralization assay and the titer of conventional HI assay, tested with 14 serum samples collected ≥10 days after symptom onset from patients with real-time RT-PCR–confirmed 2013 influenza A(H7N9) infection (○) and 50 control samples (△, Spearman r = 0.88, p<0.0001, n = 64). To display the information from all the samples, overlapped markers were shifted on the x- and/or y-axis with small incremental units.

Conclusions

Here we provide an alternative approach for quantifying antibody responses to the new avian influenza A(H7N9) virus. Conventional HI or MN assays require propagating the live subtype H7N9 virus, which is known as a lethal virus. The pseudovirus can infect in a single round, which is much safer than handling the highly virulent subtype H7N9 virus. All of these processes for the pseudovirus-based neutralization assay can be performed at routine BSL-2 settings, and most field laboratories equipped with mammalian cell culture facilities meet this biosafety requirement. Moreover, propagating avian influenza virus in embryonic eggs to high titer is labor intensive and time consuming and requires empirical judgment to interpret the results of HI and MN assays. By contrast, large amounts of pseudoviruses take only 2–3 days to produce, and using RLA as the readout of pseudovirus-based neutralization assay provides an objective means of interpreting the endpoints.

Therefore, this pseudovirus-based neutralization assay could be used as an alternative for safely conducting serologic studies in a rapid response in assessing the threat posed by subtype H7N9 virus. Of note, the pseudovirus-based assay was less sensitive than HI assay when tested by our small number of samples, indicating that some false-negative results would be observed and thus that some samples that would test positive by HI might be missed. The differences between the 2 assays could be explained by the possibility that HI and pseudovirus-based assays evaluate different components of the antibody response. HI only measures the proportion of antibodies directed to the receptor-binding site of hemagglutinin, whereas the neutralization assay detects a broader range of neutralizing antibodies. The results of these 2 assays were in agreement for most samples, indicating that 2 different components of antibodies are likely to be developed in parallel in most

Table. Comparison of HI and pseudovirus-based neutralization assay results for influenza A(H7N9) virus*

	Pseudovirus IC_{50} titer			
HI titer†	≥40	<40‡	≥20§	<20§
+	12	2	13	1
–	0	50	4	46

*Values are no. samples. HI, hemagglutination inhibition; IC_{50}, 50% inhibitory concentration.
†+ HI titer is ≥40; – HI titer is <40.
‡κ = 0.904; McNemar test, p = 0.5.
§κ = 0.788; McNemar test, p = 0.375.

persons. Therefore, this assay will provide a new contribution to the understanding of how the immune system responds to infection with influenza viruses.

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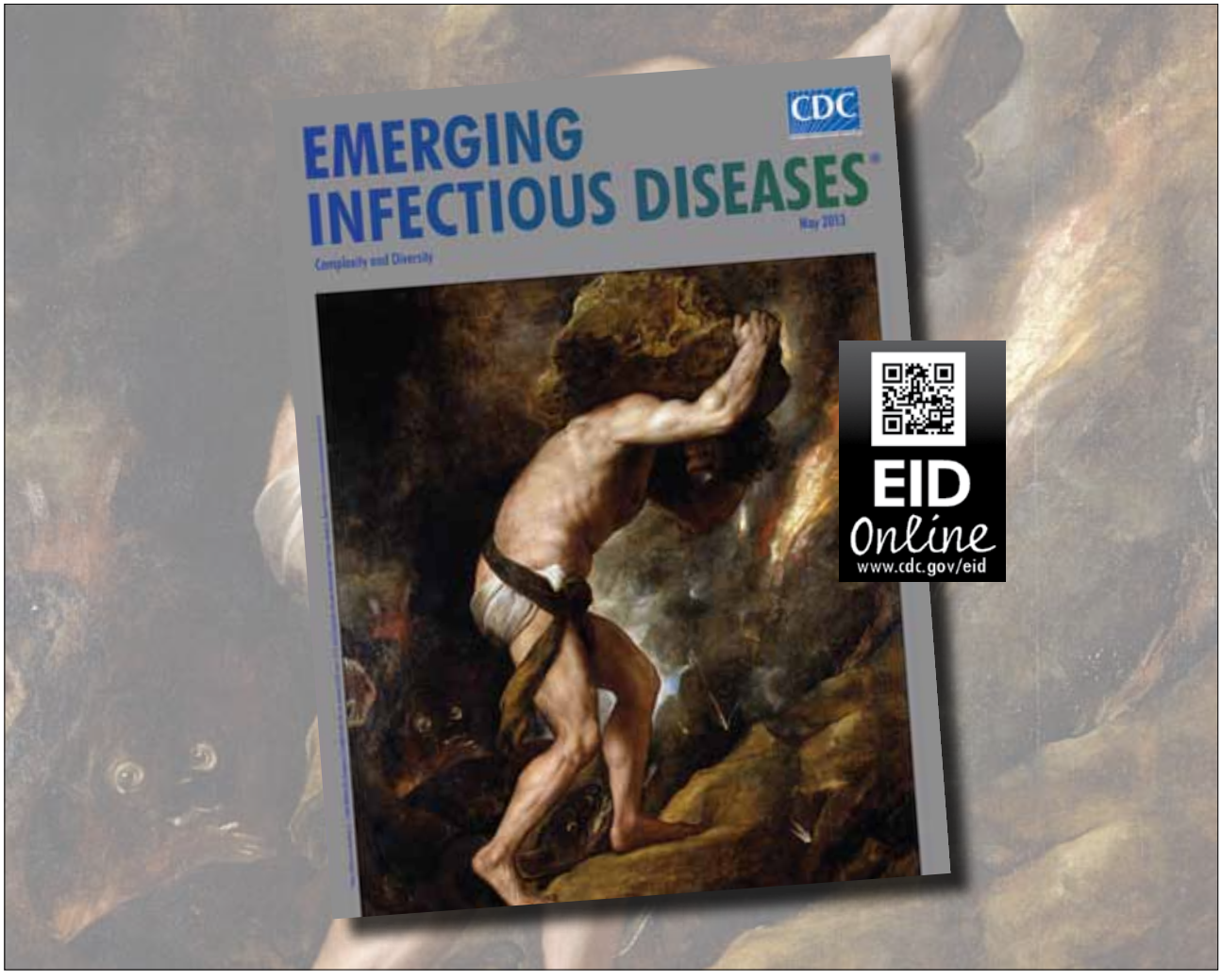

Think Fungus–Prevention and Control of Fungal Infections

Mary E. Brandt and Benjamin J. Park

Reports of human infections with environmental fungi are on the increase throughout the world. Many of these reports describe infections caused by new agents, as well as by traditional agents with new virulence factors or new mechanisms of infection. Fungal infections historically have been underrecognized and difficult to detect, and treatment options are poor.

The reasons for their emergence are likely multifactorial. The advent of medical progress–including the wide use of medical hardware, such as central lines; successful management of immunosuppression in patients with transplanted organs; and immunomodulatory agents for treating underlying diseases from cancer to rheumatoid arthritis–has contributed to the increase in fungal infections in immunocompromised hosts. Risk factors such as changes in land use, seasonal migration, international travel, extreme weather, and natural disasters, and the use of azole antifungal agents in large-scale agriculture are believed to underlie many of the increases in community-acquired fungal infections.

Because fungal infections are frequently underrecognized and difficult to detect, one of the largest gaps in our understanding of their epidemiology is determining the incidence of disease. In an article in this issue of Emerging Infectious Diseases, Sondermeyer et al. document the incidence and cost of hospitalizations in California caused by Valley fever (coccidioidomycosis), a fungal infection endemic to the southwestern United States and parts of Latin America (*1*). This article reports that during 2000–2011, there were >25,000 Valley fever–associated hospitalizations in California and >$2 billion in hospital costs. The rate of hospitalizations increased over the study period from 2.3 to 5.0 cases/100,000 population, a finding that supports other recent publications documenting the increasing incidence of Valley fever in the United States (*2*,*3*). Although the reasons for this increase are not well understood, the practical effect of this increasing incidence has been seen in many settings, including the California state prison system. Recently, a federal court ordered the prison system to move prisoners believed to be at high risk for Valley fever (including Blacks, persons >55 years of age, and persons with preexisting diabetes) out of 2 prisons in the San Joaquin Valley, which is the region in California to which coccidioidomycosis is endemic.

Fungal diseases also appear to be emerging beyond their traditionally described borders for reasons that are not entirely understood. One article in this issue reports the incidence of *Cryptococcus gattii* disease, once believed to be restricted to tropical regions, but which is now found in locations as disparate as Vancouver Island, Canada and parts of the southeastern United States (*4*). Although this organism is genetically related to *C. neoformans*, a cause of meningitis in HIV-infected persons, *C. gattii* is frequently associated with a different spectrum of disease, prominently pneumonia. In another article in this issue, Nucci et al. report an increase in incidence of community-associated *Fusarium* spp. infections in a cancer ward in Brazil (*5*). In this study, *Fusarium* spp. caused an increase in invasive infections, which usually started as skin or nail infections, in immunocompetent and immunosuppressed patients. Although the root cause was not determined, speculation has centered on changes in land use patterns and agricultural practices in Brazil. Also in this issue, a novel agent of fungemia, *Candida auris*, is reported as having been detected in India (*6*). All isolates reported in this article were resistant to the antifungal agent fluconazole, which is concerning because fluconazole is frequently the first-line treatment for invasive *Candida* spp. infections in many countries. Finally, an article by Fong et al. provides serologic evidence that *Pneumocystis jirovecii* may be transmitted between patients and providers in the health care setting, a finding that could affect future infection control policies (*7*).

Because most invasive fungal infections have high mortality rates, reducing the incidence of these diseases often relies on rapid and specific diagnostics, effective antifungal drugs, novel immunotherapeutic strategies, and adherence to infection control and sterility practices. Recently, we have seen examples of successes and failures in this area. In regions with high HIV prevalence, use of

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novel lateral-flow diagnostic tests for cryptococcal disease has opened the door to systematic screening and point-of-care testing in asymptomatic persons with low CD4 cell counts and may result in reduction of deaths caused by this disease (*8,9*). Conversely, recent contamination of a widely distributed injectable steroid medication with fungal organisms, particularly the black mold *Exserohilum rostratum*, caused the largest health care–associated outbreak in the United States; as of July 1, 2013, there have been 749 cases of meningitis and related infections among persons in 20 states and 61 deaths (*10,11*). Swift public health actions, including notification of patients and providers, led to rapid clinical assessments and institution of antifungal therapy among infected persons, thereby reducing the mortality rate and effects of this disease (*12*).

Broader control of fungal exposures in the community can also be improved by awareness, especially education regarding high-risk practices and activities. Outbreaks of histoplasmosis linked to construction and cleaning activities in places contaminated with bird or bat guano have led to production of educational materials describing how risk can be mitigated (*13*). Furthermore, recent advances in whole-genome sequencing are being explored to suggest novel vaccine and diagnostic targets for the agent of Valley fever (*14*).

Fungal infections remain serious and underappreciated causes of illness and death. Much can be done to prevent the consequences of these infections, although environmental exposure to these agents may not be entirely avoidable in the community. Continued public health efforts toward defining, characterizing, and tracking the emergence of fungal infections can help to focus studies on priority infections and settings. Future translational research is urgently needed to develop novel diagnostics, vaccines, and treatments as more is learned about the pathogenesis of fungal infections and the biology of fungal agents.

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Leptotrichia trevisanii Sepsis after Bone Marrow Transplantation

To the Editor: *Leptotrichia* spp. have been identified as the cause of various infections. However, the most commonly reported infection is bacteremia in the setting of chemotherapy for hematologic malignancies (*1*,*2*). Only recently has *L. trevisanii* emerged as a cause of infection; case reports are rare (*3*–*5*). We recently observed 3 cases of *L. trevisanii* bacteremia in patients who had recently undergone peripheral blood stem cell transplantation (SCT.) Our goal was to identify possible causes of these infections.

The patients were 2 men and 1 woman (ages 53, 56, and 63 years, respectively) who had received myeloablative chemotherapy. The 2 men had multiple myeloma and relapsed follicular non-Hodgkin lymphoma and had neutropenic fever 5 and 4 days post-SCT, respectively. The woman had acute myelogenous leukemia that had arisen from a myelodysplastic syndrome after matched sibling donor SCT failure. She had neutropenic fever on day 13 of induction therapy.

Multiple blood cultures from >1 site (peripheral and central venous catheter [CVC] or 2 separate CVCs) obtained from each patient during the initial febrile episode grew *L. trevisanii*. For the 2 patients with positive cultures for peripheral blood and CVC sites, the peripheral culture was reported as positive before the CVC culture but not before use of the CVC. All subsequent blood cultures and catheter tip cultures from these patients had negative results for bacteria.

All organisms were cultured by using the BacT/ALERT 3D blood culture instrument (bioMeriéux, Durham, NC, USA) and standard aerobic and anaerobic media. Times to positivity were approximated (range 28–58 hours). Gram staining of isolates from culture media showed large, fusiform gram-negative rods. One isolate had gram-positive beading and was reported as gram variable. A second isolate grew anaerobically from initial subculture on 5% sheep blood agar but grew aerobically in chocolate agar in 5% CO_2 on second subculture. A third isolate showed pinpoint growth on initial aerobic culture on sheep blood agar. No isolates were identified by using the RapID ANA II System (Remel, Lenexa, KS, USA).

One organism was identified as *Sphingomonas paucimobilis* by Vitek 2 (bioMeriéux), but this result was inconsistent with results of other biochemical tests. The 3 organisms were sent to the Mayo Medical Laboratories (Rochester, MN, USA) for anaerobic bacteria identification and speciation by 16S rRNA gene sequencing. All catheter tips were cultured by rolling a 1-inch segment of the catheter on sheep blood agar and incubating them aerobically in 5% CO_2 for 5 days.

The reason *L. trevisanii* has only recently been identified as a cause of bacteremia in neutropenic patients is likely multifactorial. Our findings suggest routine use of 16S rRNA gene sequencing and increased numbers of bone marrow transplants as the major reasons.

L. trevisanii was discovered in 1999. More than a decade had passed between the availability of 16S rRNA sequencing and discovery of this bacterium. (*5*). Some authors suggested that previous lack of recognition may have been caused by fastidious growth requirements, inconsistent staining, or misidentification (*3*,*4*,*6*). No recent major changes in instrumentation, subculture algorithm, or solid media had been made before we isolated this organism, and we had not previously isolated any unidentified organisms with similar appearance and growth patterns typical of *L. trevisanii*. Unlike some species of *Leptotrichia*, *L. trevisanii* grows readily on solid media when subcultured (*3*). This finding indicates an emergent pathogen rather than a previously undiagnosed cause of bacteremia.

We have seen an increase in the number of bone marrow transplants performed, but there has been no major change in myeloablative regimens. We observed 1 case of *L. trevisanii* bacteremia in each year during 2009–2011, in which our institution performed 185, 189, and 215 transplants, respectively (overall incidence 0.5 cases/100 transplants). This finding might explain why no cases were seen previously. All 3 patients had grades 1–2 mucositis, which in the presence of neutropenia, is a known risk factor for anaerobic bacteremia in patients undergoing chemotherapy for hematologic malignancies (*3*,*7*,*8*).

Bacteremia developed in the 3 patients while they were treated with levofloxacin. The 56-year-old man responded to a cephalosporin. The 63-year-old woman did not respond to a carbapenem or vancomycin but did respond to a second carbapenem. The 53-year-old man did not respond to a cephalosporin or metronidazole but became afebrile after treatment with vancomycin. These inconsistencies did not enable us to make specific therapeutic recommendations for treatment of *L. trevisanii* infection other than to report clinical resistance to levofloxacin.

Currently recommended treatment regimens for neutropenic fever do not include treatment for anaerobic infections. Some institutions have altered treatment regimens to include antimicrobial drugs, such as meropenem, because of increases in anaerobic bacteremias (*3*,*9*). We do not believe that the number of cases of anaerobic bacteremia at our institution warrants a change in treatment policy.

On the basis of our findings, we expect an increase in the number of cases of anaerobic bacteremia after an expected increase in the number of bone marrow transplants performed. Future policies include improved

treatment or prevention of mucositis, earlier detection and identification of isolates, and revision of current antimicrobial drug protocols for empiric treatment of neutropenic fever.

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Clinical Profile of Children with Norovirus Disease in Rotavirus Vaccine Era

To the Editor: After the substantial decrease in acute gastroenteritis (AGE) in children caused by rotavirus after introduction of 2 rotavirus vaccines (*1*), norovirus has become the leading cause of medically attended AGE in US children <5 years of age (*2*). We describe the clinical characteristics of norovirus disease and assessed whether rotavirus vaccine protected against norovirus AGE.

During October 2008–September 2010, the New Vaccine Surveillance Network enrolled 1,897 children <5 years of age with symptoms of AGE (≥3 episodes of diarrhea or any episodes of vomiting within 24 hours lasting ≤10 days) who came to hospitals, emergency departments, and outpatient clinics in Cincinnati, Ohio; Nashville, Tennessee; and Rochester, New York, USA, as described (*2*).

Epidemiologic, clinical, and vaccination data were systematically collected. Whole fecal specimens were obtained within 14 days of the date of visit and tested for rotavirus by using a commercial enzyme immunoassay (Rotaclone; Meridian Bioscience, Inc., Cincinnati, OH, USA) and for norovirus by using real-time reverse transcription quantitative PCR, followed by sequence analysis of positive samples (*3*,*4*). Clinical severity was assessed by using a 20-point scoring system (*5*), which was modified to use behavior as a proxy for dehydration. Odds ratios used to calculate vaccine effectiveness (VE) were adjusted for race and insurance status (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0448-Techapp1.pdf).

Inclusion criteria for this study corresponded with criteria used in previous New Vaccine Surveillance Network studies (*2*,*6*). Children were age eligible for pentavalent rotavirus vaccination (RV5), had a fecal specimen tested for norovirus and rotavirus, and had complete vaccination and AGE symptom information (online Technical Appendix Figure 1). Children who received a dose of monovalent rotavirus vaccine or vaccine of unknown type or were positive for rotavirus and norovirus were excluded from analyses. Only unvaccinated rotavirus-positive children (n = 69, 72%) were used in severity score analyses because RV5 is known to attenuate rotavirus illness (*6*).

Of the enrolled children, 574 met the inclusion criteria; 144 (25%) norovirus-positive case-patients, 96 (17%) rotavirus-positive case-patients, and 334 (58%) patients negative for norovirus and rotavirus (control patients with AGE) (online Technical Appendix Figure 1). Of 144 norovirus-positive specimens, 10 (7%) could not be genotyped, 4 (3%) were positive

for genogroup (G) I, and 130 (90%) were positive for GII. The most common genotype was GII.4 Minerva (74 [51%]).

Norovirus case-patients were significantly more likely than control patients with AGE to have longer duration and more episodes of vomiting in a 24-hour period (p = 0.003 and p<0.0001, respectively) but were significantly less likely to report fever (p = 0.001) (Table). However, the median severity score for norovirus case-patients did not differ from that for control patients with AGE (11 vs. 10, respectively). Individual severity score components and overall severity scores did not differ among case-patients infected with norovirus who received 0, 1 or 2, or 3 doses of RV5, but the duration of vomiting was longer in case-patients infected with norovirus GII.4 than in those infected with a non-GII.4 genotype (online Technical Appendix Tables 1, 2; Figure 2).

Relative to the 69 unvaccinated rotavirus case-patients, norovirus case-patients had shorter duration and fewer episodes of diarrhea in a 24-hour period (p = 0.003 and p = 0.0003, respectively). Norovirus case-patients were also significantly less likely to be hospitalized (p = 0.02), have fever (p<0.0001), and have severe behavior changes (p<0.0001); they also had lower overall severity scores (p<0.0001) than unvaccinated rotavirus case-patients.

Compared control patients with AGE, VE of any dose of RV5 against norovirus disease was –0.9% (95% CI –55% to 34%). A full course of RV5 likewise showed no evidence of protection against norovirus (VE 5%; 95% CI –50% to 40%), and results were consistent across age groups.

In conclusion, we found that norovirus AGE was associated with more frequent and prolonged vomiting but less fever than AGE not caused by norovirus or rotavirus. Case-patients infected with norovirus GII.4 also had a longer duration of vomiting than did case-patients

Table. Clinical profile and severity score of norovirus case-patients compared with AGE control patients and unvaccinated rotavirus case-patients, New Vaccine Surveillance Network, United States, 2008–2010*

Severity score component	Severity score	Norovirus case-patients, n = 144	Unvaccinated rotavirus case-patients, n = 69	p value†	AGE control patients, n = 334	p value†
Duration of diarrhea, d, no. (%)				**0.003**		0.19
0	0	32 (22)	3 (4)		82 (25)	
1–4	1	87 (60)	55 (80)		171 (51)	
5	2	13 (9)	7 (10)		33 (10)	
≥6	3	12 (8)	4 (2)		48 (14)	
Diarrhea episodes/24 h, no. (%)				**0.0003**		0.24
0	0	32 (22)	3 (4)		82 (25)	
1–3	1	47 (33)	16 (23)		79 (24)	
4–5	2	22 (15)	14 (20)		64 (19)	
≥6	3	43 (30)	36 (52)		23 (33)	
Duration of vomiting, h, no. (%)				0.43		**0.003**
0	0	7 (5)	2 (3)		54 (16)	
1–23 (1 d)	1	28 (19)	10 (14)		64 (19)	
24–47 (2 d)	2	33 (23)	12 (17)		74 (22)	
≥48 (≥3 d)	3	76 (53)	45 (65)		142 (43)	
Vomiting episodes/24 h, no. (%)				0.22		**<0.0001**
0	0	7 (5)	2 (3)		54 (16)	
1	1	11 (8)	1 (1)		52 (16)	
2–4	2	45 (31)	20 (29)		117 (35)	
≥5	3	81 (56)	46 (67)		111 (33)	
Fever, °F, no. (%)				**<0.0001**		**<0.0001**
≤98.6	0	80 (56)	15 (22)		102 (31)	
98.7–101.1	1	29 (20)	21 (30)		55 (16)	
101.2–102	2	9 (6)	18 (26)		45 (13)	
≥102.1	3	26 (18)	15 (22)		132 (40)	
Behavioral signs, no. (%)				**<0.0001**		0.65
Normal	0	12 (8)	2 (3)		35 (10)	
Less playful/irritable	1	63 (44)	13 (19)		158 (47)	
Lethargic/listless	2	67 (47)	54 (78)		138 (41)	
Seizure	3	2 (1)	0 (0)		3 (1)	
Treatment, no. (%)				**0.02**		0.16
None	0	50 (35)	12 (17)		135 (40)	
Rehydration, no hospitalization	1	50 (35)	26 (38)		87 (26)	
Hospitalization	2	44 (31)	31 (45)		112 (34)	
Severity score, median	NA	11	13	**<0.0001**	10	0.78

*AGE control patients were those who had AGE (defined as ≥3 episodes of diarrhea or any episodes of vomiting within 24 h that lasted ≤10 d) but who were negative for norovirus and rotavirus. AGE, acute gastroenteritis; NA, not applicable.
†Severity scores were compared by Wilcoxon rank-sum test. All other components were compared by Fisher χ^2 test. Significant findings are indicated in **boldface**.

infected with non-GII.4 norovirus genotypes. However, AGE among unvaccinated rotavirus case-patients was more severe than among norovirus case-patients, and was characterized by higher fever and more frequent and severe diarrhea. This finding confirms findings in a study of children in Finland (*7*), although our study found no difference in frequency or severity of vomiting between patients with rotavirus disease and those with norovirus disease.

In addition, vaccination against rotavirus did not provide protection against norovirus and had no effect on the clinical course of norovirus disease, which is consistent with other findings (*8*). Although an earlier rotavirus vaccine, which has subsequently been withdrawn, may have provided some nonspecific protection by reducing intensity and duration of diarrhea associated with adenovirus and sapovirus (*9*,*10*), our study did not demonstrate a similar effect on norovirus-associated diarrhea after vaccination with RV5. This study reinforces the hypothesis that norovirus can cause severe AGE among young children and should be considered as a specific target for vaccine development.

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Leprosy in Pregnant Woman, United States

To the Editor: Hansen disease, or leprosy, in pregnancy is a rarely reported event in the United States. In 2009, a total of 213,036 new cases of leprosy were detected througout the world (*1*). Nine countries in Africa, Asia, and Latin America consider it a public health problem, accounting for ≈75% of the global disease prevalence (*1*).

We describe a case of leprosy in a 27-year-old woman with 1 previous pregnancy and 1 live-born infant who had onset of subcutaneous nodules before she became pregnant. She appeared at her initial prenatal visit at 24.1 weeks of gestation after recently emigrating from Mexico. The patient reported that subcutaneous nodules had developed on her arms, legs, back, and abdomen ≈5 months before the visit, 2 weeks before her last menstrual period. A skin biopsy revealed acute and chronic panniculitis with acid-fast bacilli, and the condition was confirmed by PCR to be lepratamatous leprosy. Treatment included rifampin, Dapsone, clofazimine, and prednisone.

The patient's condition was monitored closely with ultrasounds at serial intervals; these showed consistent fetal growth at the 50th percentile. At 37 weeks and 1 day, her membranes

ruptured. She underwent a repeat cesarean delivery because the method of leprosy transmission is not yet proven and to prevent possible vertical transmission to the infant. The patient delivered a female infant weighing 6 lb, 8 oz, with Apgar scores of 8 and 9. On postoperative day 1, Dapsone treatment was restarted; she was given Dapsone, 50 mg daily, and prednisone, 40 mg daily. She was discharged with the baby on postoperative day 3.

Leprosy is a chronic disease caused by *Mycobacterium leprae*. The disease mainly affects the skin and nerves and, if untreated, can cause permanent damage. It is curable, however, and disability can be avoided. The World Health Organization recommends multidrug therapy consisting of Dapsone, rifampin, and clofazimine (*1*). This combination has proven highly effective, and patients are no longer infectious after the first dose (*1*). Virtually no relapses occur and antimicrobial drug resistance does not develop (*1*). Pregnancy causes a relative decrease in cellular immunity, which allows *M. leprae* to proliferate (*2*). Careful management can prevent permanent nerve damage. Lepromatous leprosy and relapse after treatment are more commonly seen throughout pregnancy because of the pregnant woman's immunodeficient state (*2,3*). Infants are usually less affected than mothers; nevertheless, selection of the mother's antimicrobial drug regimen must ensure adequate control of the bacteria while avoiding teratogenicity and in utero adverse effects, such as low birthweight (*3*,*4*). The infant has a potentially high risk of contracting leprosy from the mother by skin-to-skin contact or droplet transmission, particularly if she has not received treatment.

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Haemophilus parahaemolyticus Septic Shock after Aspiration Pneumonia, France

To the Editor: Members of the genus *Haemophilus* are commensal bacteria of the upper respiratory tract, and *H. influenzae* is the main pathogen in this genus that can cause a wide range of human infections (*1*). The species most closely related to *H. influenzae* is *H. haemolyticus*, usually considered a commensal of the nasopharynx in humans; it can be pathogenic, although rarely (*2*,*3*). *H. parahaemolyticus* was distinguished from *H. haemolyticus* in 1953 when it was determined that *H. parahaemolyticus* required only factor V, but not factor X, for growth (*4*). This species could be responsible for pharyngitis and, rarely, for subacute endocarditis (*4*), but it has seldom been reported to cause invasive disease (*5*). Invasive disease has been reported in a patient who had an empyema in the gallbladder (*6*) and in a patient who had a cryptogenic brain abscess (*7*). We report a case of acute respiratory distress syndrome (ARDS) and septic shock caused by *H. parahaemolyticus*.

A 50-year-old woman, who was receiving artificial ventilation, was transferred to the Hôpital Nord in Marseille, France, in November 2012. She was in a coma because she had taken an overdose of benzodiazepine and clomipramine in a suicide attempt. She had no relevant medical history except addiction to tobacco, chronic alcoholism, and depression. The patient's family owned 3 cats and 1 dog but had no other pets. She had not traveled outside France.

Notable findings on initial examination (before intubation) were shock (arterial blood pressure 80/50 mm Hg, despite adequate fluid resuscitation), tachycardia (pulse rate 110 beats/min), tachypnea (respiratory rate 34 respirations/min), low peripheral oxygen saturation (S_pO_2 80%), and a temperature of 39°C. On intensive care unit (ICU) admission, her partial pressure of arterial oxygen/fraction of inspired oxygen ratio was 101 under mechanical ventilation, with a positive end-expiratory pressure level of 10 cm H_2O, consistent with ARDS. Chest examination revealed bilateral crackles on lower lung lobes, consistent with chest radiograph findings. Routine laboratory evaluation showed a hemoglobin level of 11.7 g/dL (reference 12.5–15.5 g/dL for women); leukocytosis, indicated by a leukocyte count of 23 cells $\times 10^9$/L (reference 4–10 $\times 10^9$/L); and elevated C-reactive protein level (298 mg/L [reference <5 mg/L]).

On the second day, a bronchoalveolar lavage (BAL) specimen (obtained before initiation of antimicrobial drug therapy) was positive for *H. parahaemolyticus* (10^7 CFU/mL) on a chocolate polyvitex agar plate, and the strain showed susceptibility to ampicillin/clavulanate, ceftriaxone,

gentamicin, and ciprofloxacin. The bacterium was hemolytic and required factor V, but not factor X, for growth. The bacterium was identified in the laboratory by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry with the Bruker Biotyper software database (Bruker Daltonics, Bremen, Germany), with a good score (>2.0) (*8*). The identification was confirmed by PCR amplification and sequencing of the 16S rRNA gene (size of sequence was 1,387 bp, and it had 99.6% homology with sequence AJ295746 in GenBank). Blood culture results were negative for both bacterial species. The final diagnosis was septic shock associated with ARDS, due to aspiration pneumonia.

In the ICU, the patient received ampicillin/clavulanate and gentamicin, along with vasopressor therapy and crystalloid fluid resuscitation. Her response was dramatic, and her condition improved rapidly. When she was stabilized and able to take oral drugs, she was given ampicillin/clavulanate, 1 g 3× daily for 7 days. On day 9, she was discharged from the ICU in stable condition. One month after discharge, she attended a follow-up visit at the pulmonary outpatient department and had made a full recovery.

This study shows the isolation in pure culture of *H. parahaemolyticus* from the BAL specimen of a patient with septic shock with ARDS. The isolate was unambiguously identified by MALDI-TOF (*8*) and confirmed by 16S rRNA sequencing. Correct identification of bacteria of the genus *Haemophilus* at the species level, including *H. parahaemolyticus*, by MALDI-TOF, has also been reported in 2 recent works (*9*,*10*). Isolation of this bacterium in pure culture from the BAL specimen was eventually associated with the disease of the patient (including a coma complicated with aspiration pneumonia and bilateral pulmonary consolidations), and the patient rapidly improved after receiving antimicrobial drug treatment.

These findings suggest that *H. parahaemolyticus* was the causative agent of the patient's disease. Although this bacterium has been rarely reported as a cause of human infections, it should be considered as an opportunistic pathogen, especially in patients who have aspiration pneumonia, because it is likely a commensal of the upper respiratory tract. Among 31 *H. parahaemolyticus* isolates from human specimens reported by Nørskov-Lauritsen et al., 75% of the isolates were recovered as commensals in the pharynx and throat and from sputum (*10*). This bacterium was likely overlooked in the past because phenotypic identification was not sufficiently accurate to distinguish it from other *Haemophilus* spp. Thus, *H. parahaemolyticus* has a pathogenic potential for causing invasive and severe diseases in humans that should be further investigated.

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Mycobacterium iranicum Infection in HIV-infected Patient, Iran

To the Editor: The species *Mycobacterium iranicum* was described in 2013 (*1*) on the basis of 8 clinical strains isolated in various countries (Iran, Italy, Greece, the Netherlands, Sweden, and the United States). Recently, the isolation of *M. iranicum* from the sputum of a woman also was reported (*2*). We report the isolation of this newly recognized species from an HIV-positive patient.

A scotochromogenic, rapidly growing strain was isolated in 2012 from respiratory specimens of an HIV-positive 44-year-old Iranian man with chronic pulmonary disease. The patient had been found to be HIV seropositive (viral load ≥1,000 copies/mL, CD4 lymphocyte count 120/μL) in 2004 when he was hospitalized because of fever, weight loss, and oral candidiasis. Treatment with antiretroviral drugs, including stavudine, lamivudine, and nevirapine, was begun. The patient rapidly improved; the fever disappeared, he gained weight, and he was discharged from the hospital. At a 6-month follow-up visit, viral load was 1,000 copies/mL and CD4 lymphocyte count was 420/μL. He continued to receive antiretroviral treatment until 2010 when treatment was discontinued because of its high cost.

The man was hospitalized again in 2012 with mild fever, weight loss, chronic chest pain, and nonproductive cough. At that time, the viral load was ≥100,000 copies/mL, and CD4 count was 5 lymphocytes/μL. Tuberculin skin test results were negative, radiograph of the chest showed no abnormalities, and routine cultures of sputum and blood were negative for common bacteria. Lactate dehydrogenase level (98 U/L [reference <600 U/L]) was within normal limits, whereas liver function was abnormal (alanine aminotransferase level 95 U/L [reference <36 U/L], L-aspartate aminotransferase level 85 U/L [reference <29 U/L], alkaline phosphatase 180 U/L [reference 44–147 U/L], and total bilirubin 1.4 mg/dL [reference 0.3–1mg/dL]). Antiretroviral therapy was resumed, which led to an increase in CD4 cells (205 lymphocytes/μL after 1 month). The examination by microscopy (Ziehl-Neelsen staining) of 3 sputum samples did not reveal acid-fast bacilli; culture for mycobacteria was not done. Oral treatment with tetracycline was started, but the patient's fever and chest pain remained unchanged.

After bronchoscopy, 2 of 3 bronchial lavage (BAL) samples were found to be positive for acid-fast coccobacilli by microscopy, and rapidly growing, deep orange mycobacteria grew in all 3 cultures. Giemsa stain did not show *Pneumocystis jirovecii* in BAL samples. A standard antituberculosis regimen was undertaken but did not result in substantial improvement. At 1 month follow-up, 1 sputum sample was negative for acid-fast organisms, and 1 BAL specimen was positive by microscopy and in culture. When the isolate was identified as *M. iranicum*, therapy was replaced with a combination of amikacin and ciprofloxacin for 3 months (standard treatment used in Iran for infections caused by rapidly growing mycobacteria), and the patient improved rapidly. Mycobacteria were neither observed nor grew in culture in a BAL specimen obtained 1 month after the change in therapeutic regimen.

Identification of the isolates was initially attempted with biochemical tests, and they were negative for niacin production, nitrate reduction, Tween 80 hydrolysis, and semiquantitative catalase. The tests were positive for urease activity, iron uptake, tellurite reduction, arylsulfatase (3 days after the start of the test), 5% NaCl tolerance, and heat-stable (68°C) catalase. The genetic sequencing of almost-complete (1,450 bp) 16S rRNA gene (*3*), a 710-bp fragment of the β-subunit of the RNA polymerase gene (*4*), and the hypervariable region (402 bp) of the 65-kDa heath-shock protein (*5*) revealed 99.8%, 99.4%, and 100% identity, respectively, with sequences of the type strain found in GenBank and definitively confirmed the identification.

The clinical criteria required by the American Thoracic Society and Infectious Disease Society of America (*3*) to assess the importance of the isolation of a nontuberculous mycobacterium from pulmonary specimens include, in adjunct to a specific symptomatology, the presence of nodular or cavitary lung lesions and the exclusion of any other possible cause of the disease. The normal thoracic radiograph findings for the case-patient described here cannot, however, be considered a definitive exclusion criterion: in highly immunocompromised patients, a chest radiograph may show no abnormalities, even when substantial pathologic features of infection are present (*6*). The microbiological criteria were clearly fulfilled by isolating the organism from multiple sputum specimens and the BAL specimens. The patient's response to the treatment and the disappearance of thoracic symptoms further support the assertion.

Our report confirms the potential pathogenicity of *M. iranicum*. In addition to the case described here, 9 isolations of this species have been reported so far. Among them, the clinical relevance has been demonstrated for 2 strains grown from respiratory specimens of patients with pulmonary disease and for 1 strain isolated from a cutaneous lesion (*1,2*). The role of an accurate identification, in conjunction with symptoms and radiographic findings, is central to understanding the clinical significance of mycobacteria isolated from pathologic specimens.

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Close Relative of Human Middle East Respiratory Syndrome Coronavirus in Bat, South Africa

To the Editor: The severe acute respiratory syndrome (SARS) outbreak of 2002–03 and the subsequent implication of bats as reservoir hosts of the causative agent, a coronavirus (CoV), prompted numerous studies of bats and the viruses they harbor. A novel clade 2c betacoronavirus, termed Middle East respiratory syndrome (MERS)–CoV, was recently identified as the causative agent of a severe respiratory disease that is mainly affecting humans on the Arabian Peninsula (*1*). Extending on previous work (*2*), we described European *Pipistrellus* bat–derived CoVs that are closely related to MERS-CoV (*3*). We now report the identification of a South Africa bat derived CoV that has an even closer phylogenetic relationship with MERS-CoV.

During 2011–2012, fecal pellets were collected from 62 bats representing 13 different species in the KwaZulu-Natal and Western Cape Provinces of South Africa and stored in RNA*later* solution (Life Technologies, Carlsbad, CA, USA). Details about the bat sample are available in the online Technical Appendix Table (wwwnc.cdc.gov/EID/article/19/10/13-0946-Techapp1.pdf). RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Screening for CoVs was done by nested reverse transcription PCR using broadly reactive oligonucleotide primers targeting a conserved region in the *RNA-dependent RNA polymerase* (*RdRp*) gene (online Technical Appendix). PCR results were positive for 5 (8%) of the 62 specimens. PCR amplicons for 4 positive specimens yielded alphacoronavirus sequences related to recently described bat alphacoronaviruses from South Africa (*4*). The other positive specimen, termed PML/2011, was from an adult female *Neoromicia* cf. *zuluensis* bat sampled in 2011; the specimen yielded a novel betacoronavirus (GenBank accession no. KC869678). Online Technical Appendix Figure 1 shows the distribution of this bat species.

To obtain better phylogenetic resolution, we extended the 398-nt *RdRp* fragment generated by the screening PCR to 816 nt, as described (*5*). PML/2011 differed from MERS-CoV by only 1 aa exchange (0.3%) in the translated 816-nt *RdRp* gene fragment. Thus, PML/2011 was much more related to MERS-CoV than any other known virus. The amino acid sequence of the next closest known relatives of MERS-CoV, from European *Pipistrellus* bats (*3*), differed from MERS-CoV by 1.8%. The amino acid sequences of viruses from *Nycteris* bats in Ghana (*3*) and the 2c prototype bat CoVs, HKU4 and HKU5, from China (*6*) differed by 5.5%–7.7% from MERS-CoV. The smaller 152- to 396-nt *RdRp* fragments of 2c bat CoVs from a *Hypsugo savii* bat in Spain (*7*), bat guano in Thailand (*8*), and a *Nyctinomops* bat in Mexico (*9*) showed no or only partial overlap with the 816-nt fragment generated in this study; thus, a direct comparison could not be done. However, in their respective *RdRp* fragments, these CoVs yielded amino acid sequence distances of 3.5%–8.0% and were thus probably more distant from MERS-CoV than the virus described here.

A Bayesian phylogenetic analysis of the 816-nt *RdRp* sequence confirmed the close relationship between PML/2011 and MERS-CoV (Figure). Their phylogenetic relatedness was as close as that of SARS-CoV and the most closely related bat coronavirus known, Rs672 from a *Rhinolophus sinicus* bat (Figure). Like PML/2011 and MERS-CoV, Rs672 and SARS-CoV showed only 1 aa exchange in

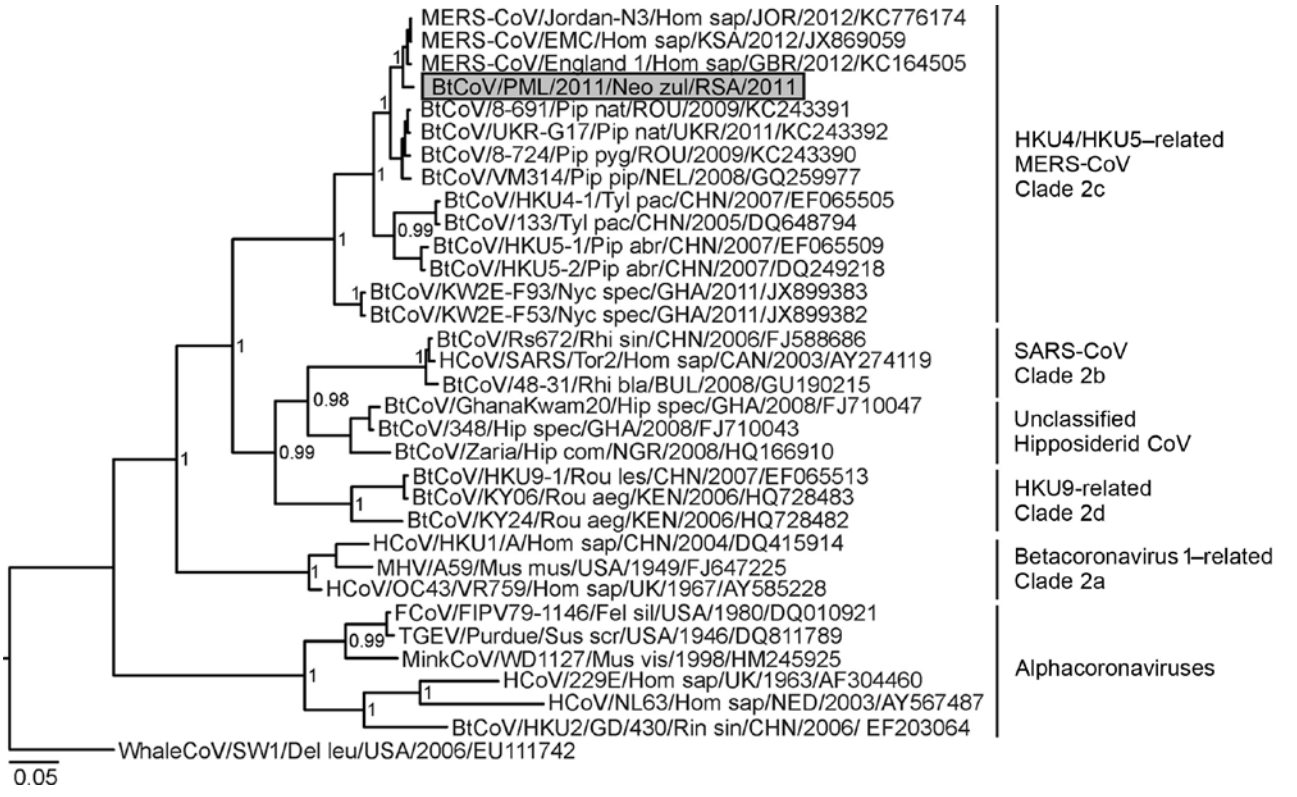

Figure. Partial RNA-dependent RNA polymerase (*RdRp*) gene phylogeny, including the novel betacoronavirus from a *Neoromicia zuluensis* bat in South Africa (GenBank accession no. KC869678 for both partial *RdRp* and *spike* gene sequences). The Bayesian phylogeny was done on a translated 816-nt *RdRp* gene sequence fragment, as described (*5*). MrBayes V3.1 (http://mrbayes.sourceforge.net/) was used with a WAG substitution model assumption over 2,000,000 generations sampled every 100 steps, resulting in 20,000 trees, of which 25% were discarded as burn-in. A whale gammacoronavirus was used as an outgroup. The novel *N. zuluensis* bat virus is highlighted in gray. Values at deep nodes represent statistical support from posterior probabilities. Only values >0.9 are shown. Coronavirus clades are depicted to the right of taxa. Scale bar represents genetic distance. MERS-CoV, Middle East respiratory syndrome coronavirus; SARS, severe acute respiratory syndrome; Bt-CoV, bat coronavirus; HCoV, human coronavirus, MHV, mouse hepatitis virus; FCoV, feline coronavirus; TGEV, transmissible gastroenteritis coronavirus.

the translated 816-nt *RdRp* fragment. To confirm this relatedness, we amplified and sequenced a short 269-nt sequence encompassing the 3′-terminus of the *spike* gene for PML/2011 (oligonucleotide primers available upon request from the authors). A partial *spike* gene–based phylogeny using this sequence yielded the same topology as that using the partial *RdRp* sequence (online Technical Appendix Figure 2). Again, PML/2011 was most closely related to MERS-CoV, showing only a 10.9% aa sequence distance in this gene, which encodes the glycoprotein responsible for CoV attachment and cellular entry. This distance was less than the 13.3% aa sequence distance between MERS-CoV and the European *Pipistrellus* CoVs (*3*) and less than the 20.5%–27.3% aa sequence distance between MERS-CoV and HKU5 and between MERS-CoV and HKU4 (*6*) in the same sequence fragment.

Our results further support the hypothesis that, like human CoV-229E and SARS-CoV, ancestors of MERS-CoV might exist in Old World insectivorous bats belonging to the family Vespertilionidae, to which the genera *Neoromicia* and *Pipistrellus* belong (*3*). Knowledge of the close relatedness of PML/2011 and MERS-CoV, which contrasts with the more distant relatedness of CoVs in bats from the Americas and Asia, enables speculations of an African origin for bat reservoir hosts of MERS-CoV ancestors. This hypothesis is limited by a global sampling bias, the small sample size, and the single clade 2c betacoronavirus detection in this study. Still, a putative transfer of MERS-CoV ancestors from Africa to the Arabian Peninsula would parallel the transfer of other viruses (e.g., the exportation of Rift Valley fever virus from East Africa, which led to a severe outbreak in Saudi Arabia in 2000) (*10*).

Studies of Vespertilionidae bats and potential intermediate hosts (e.g., carnivores and ungulates, such as camels) are urgently needed to elucidate the emergence of MERS-CoV. Such studies should focus on the Arabian Peninsula and Africa.

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Multidrug-Resistant *Escherichia coli* Bacteremia

To the Editor: Extraintestinal pathogenic *Escherichai coli* (ExPEC) bacteria have the ability to cause diverse and serious diseases, such as urinary tract infections (UTIs) and bacteremia (*1*–*3*); incidence of bacteremia is increasing globally (*4*). The emergence of multidrug resistance in *E. coli* is also becoming a global concern, with particular emphasis on *E. coli* sequence type (ST) 131, which is being increasingly reported in UTIs. Drug resistance is mediated by extended-spectrum β-lactamases (ESBLs), mainly of the CTX-M family, particularly CTX-M-15 and 14, and less frequently of the SHV and OXA families (*5*,*6*). Few studies are available regarding the characterization of *E. coli* strains causing bacteremia.

We characterized 140 *E. coli* isolates from bacteremia patients treated at Nottingham University Hospital (Nottingham, UK) over a 5-month period, with the aim of developing an epidemiologic profile of the population of ExPEC that causes bacteremia. For context, we compared the isolates with 125 *E. coli* isolates from urine samples collected during the same period. Cases were selected to include isolates from a diverse patient group: patient ages ranged from 1 month to 90 years; patient sex was evenly divided between male and female; infections were community- and hospital-associated; and suspected sources of infection varied. Antimicrobial drug susceptibility tests, PCR detection of ESBL genes, multilocus sequence typing using the Achtman scheme (http://mlst.ucc.ie/mlst/dbs/Ecoli), and virulence-associated gene (VAG) carriage screening by PCR were performed on isolates as described (*7*).

Significantly more bacteremia *E. coli* isolates than urine *E. coli* isolates were resistant to ciprofloxacin (25.7%

vs. 8.8%; p≤0.001) and cefradine (20.0% vs. 11.2%; p≤0.05). These results were reflected in the number of isolates in the 2 populations displaying a multidrug- resistance phenotype (resistance to antimicrobial drugs belonging to ≥2 classes); a significantly higher number of multidrug-resistant bacteremia *E. coli* isolates than multidrug-resistant urine isolates were found (50.7% vs. 32%; p = 0.01). PCR screening for ESBL carriage showed significantly higher ESBL carriage in bacteremia *E. coli* isolates than urine isolates for bla_{SHV} (15.7% vs. 5.6%; p = 0.008), $bla_{CTX\text{-}M}$ (29.3% vs. 17.6%; p = 0.025), and bla_{OXA} (14.3% vs. 6.4%; p = 0.037). Total ESBL carriage for bacteremia isolates was also significantly higher than for urine isolates (59.3% vs. 29.6%; p≤0.001).

Multilocus sequence types were determined for all *E. coli* isolates. A total of 63 STs were found among the urine isolates (Figure, panel A); the highest prevalence was ST73 (n = 16, 12.8%), followed by ST131 (n = 9, 7.2%), ST69 (n = 9, 7.2%), ST95 (n = 6, 4.8%), ST404 (n = 6, 4.8%), ST127 (n = 4, 3.2%), ST141 (n = 4, 3.2%), and ST10 (n = 3, 2.4%). Prevalence patterns of STs among bacteremia *E. coli* isolates were noticeably different (Figure, panel B). Three main STs were obtained. ST131 dominated (n = 30, 21.43%) and was significantly higher in prevalence than for the urine isolates (p≤0.001). ST73 (n = 24, 17.14%) and ST95 (n = 13, 9.29%) were the other 2 primary STs found. The 8 most prevalent STs in the bacteremia isolates represented 59.29% of the total population, whereas the 8 most prevalent STs in the urine isolates represented 45.6% of the total population. This finding is suggestive of selection of a smaller number of dominant STs in bacteremia.

ESBL carriage was mapped onto minimum-spanning trees for the 2 isolate groups. ESBL carriage among urine isolates was focused on a small number of STs; 19 (30.16%) of the 63 STs contained ESBL-positive isolates (Figure, panel A). The predominant ST73 group contained 18.75% ESBL-positive isolates; the other predominant STs exhibited ESBL-positive isolates at the following levels: ST131 (44.44%), ST69 (33.33%), ST95 (50%), and ST10 (0%). In contrast, 30 (51.72%) of the 58 STs among bacteremia isolates contained ESBL-positive isolates, significantly higher than for the urine isolates (p = 0.016). At the ST level, predominant STs had higher ESBL carriage in the bacteremia isolates than in the urine isolates: ST131 (50%), ST73 (50%), ST12 (75%), ST10 (100%), ST14 (50%), ST2278 (33.33%). ST95 (46.15%) and ST69 (20%) showed comparable levels. These results suggest that ESBL drug resistance is selecting for dominant ExPEC bacteremia strains.

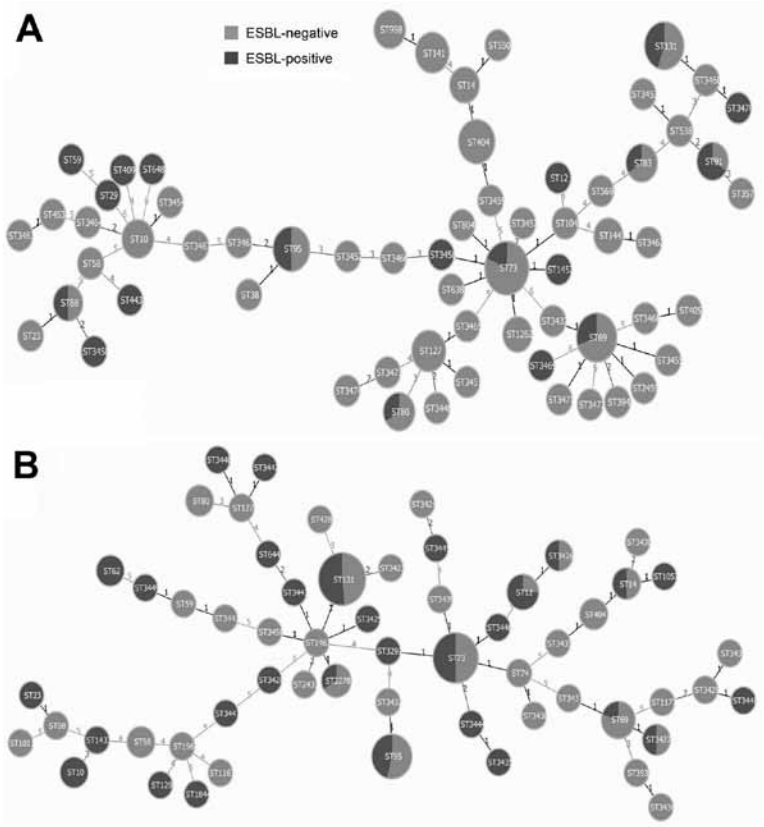

Figure. Minimum-spanning trees showing carriage of extended-spectrum β-lactamases (ESBL) in *Escherichia coli* isolates from urine samples (A) and samples from patients with bacteremia (B). Each circle represents 1 sequence type (ST), and the size of the circle reflects the number of isolates belonging to this particular ST within the bacteria group. Lines between the circles represent how different their allelic profiles are; a line labeled 1 means the linked STs differ in ≥1 of the 7 alleles, which is named a single locus variant (SLV). A cluster of STs linked by SLVs is a clonal complex. Nineteen (30.16%) of 63 STs found among the urine isolates were ESBL positive, in comparison to 30 (51.72%) of 58 for the bacteremia isolates.

To investigate whether the differences in ST observations between bacteremia and urine isolates could be attributable to differences in virulence genes, VAGs of all isolates were screened by multiplex PCR. VAGs were found equally distributed across the 2 populations, with no statistically significant difference ($p = 0.675$). Comparison of serum resistance levels between urine and blood isolates also showed no phenotypic differences.

In conclusion, we found high levels of ESBL carriage and multidrug resistance in ExPEC isolates that cause bacteremia. A comparison with urine isolates provided evidence that ESBL-mediated drug resistance appears to be the selective pressure in the emergence of dominant STs in bacteremia. Future research should focus on identifying if prolonged antimicrobial drug treatment in bacteremia patients is leading to this selection.

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Transmission of Schmallenberg Virus during Winter, Germany

To the Editor: Schmallenberg virus (SBV), an orthobunyavirus, emerged in northern Europe in 2011 (*1*). SBV infection causes transient fever, diarrhea, and a reduced milk yield in adult ruminants but, most notably, stillbirths and severe malformations in lambs and calves (*2*). Insect vectors play an essential role in transmission; the viral genome has been detected in various field-collected biting midges (*Culicoides* spp.) (*3*,*4*).

During autumn 2012 and winter 2012–2013, blood samples were taken at several times from individual sheep on a farm located in the German federal state of Mecklenburg–Western Pomerania. The farm is surrounded by agricultural fields and meadows. Approximately 1,000 ewes and their lambs, a dog, and some cats were kept on the farm; most of the animals are outdoors year-round. Only dams with ≥2 lambs are housed in open stabling in December and January. The dung is regularly cleared away and stored ≈10 m from 1 of the stable entrances. Repellents or insecticides were not applied in the monitored period. Blood samples were taken in September 2012 and in January and February 2013 and analyzed by an SBV-specific real-time quantitative reverse transcription PCR (RT-qPCR) (*5*) and by an SBV antibody ELISA (ID Screen Schmallenberg virus Indirect; IDvet; Montpellier, France) by using the recommended cutoff of 50% relative optical density as compared with the positive control (sample-to-positive ratio [S/P]).

In September 2012, blood samples from 60 sheep tested negative by the SBV antibody ELISA. Moreover, fetal malformations of the brain, spinal cord, or skeletal muscle, which might have suggested a previous SBV-infection of the dam, were not

observed during the lambing season in December 2012.

On January 10, 2013, blood samples were taken from 15 sheep that had not previously been tested; samples from all animals tested negative by ELISA. However, 4 sheep (S01–S04) tested positive by RT-qPCR (quantification cycle values: S01: 31.6, S02: 39.9, S03: 37.6, and S04: 34.9). Four weeks later, antibodies against SBV could be detected. Each of the PCR-positive blood samples was injected into 2 adult type I interferon receptor-knockout mice on a C57BL/6 genetic background. Both mice that had received blood samples of sheep S01 were seropositive after 3 weeks (S/P: 207.0 and 207.2), which demonstrates the presence of infectious virus in the inoculated blood. Assuming that viral RNA remains in the blood for just a few days, as reported after experimental infection with SBV (*1*,*6*), the sheep tested in this study had most likely been infected in early 2013. During this period, the lowest temperatures rose above 5°C for several consecutive days, with a maximum of ≈9°C (Figure, panel A). Within this brief interval, when the temperature was higher, some biting midges (*Culicoides* spp.) become active (*7*). Indeed, at the end of January, a single female biting midge (Obsoletus complex) was caught in a trap equipped with ultraviolet light; the midge tested negative by the SBV-specific RT-qPCR.

On January 23 and February 20, 2013, blood samples were taken from 90 sheep that had not previously been tested (Figure 1, panel A). A viral genome was not detected in any animal at any time. However, antibodies were detectable in 9 animals on the first sampling day. In 2 additional sheep, the S/P was in the inconclusive range; 1 of the animals tested positive after 4 weeks. In the remaining 79 sheep, no SBV antibodies could be detected; after 4 weeks, 76 sheep still tested negative by ELISA. However, the S/P of 1 sheep had increased to the inconclusive range, and 2 sheep

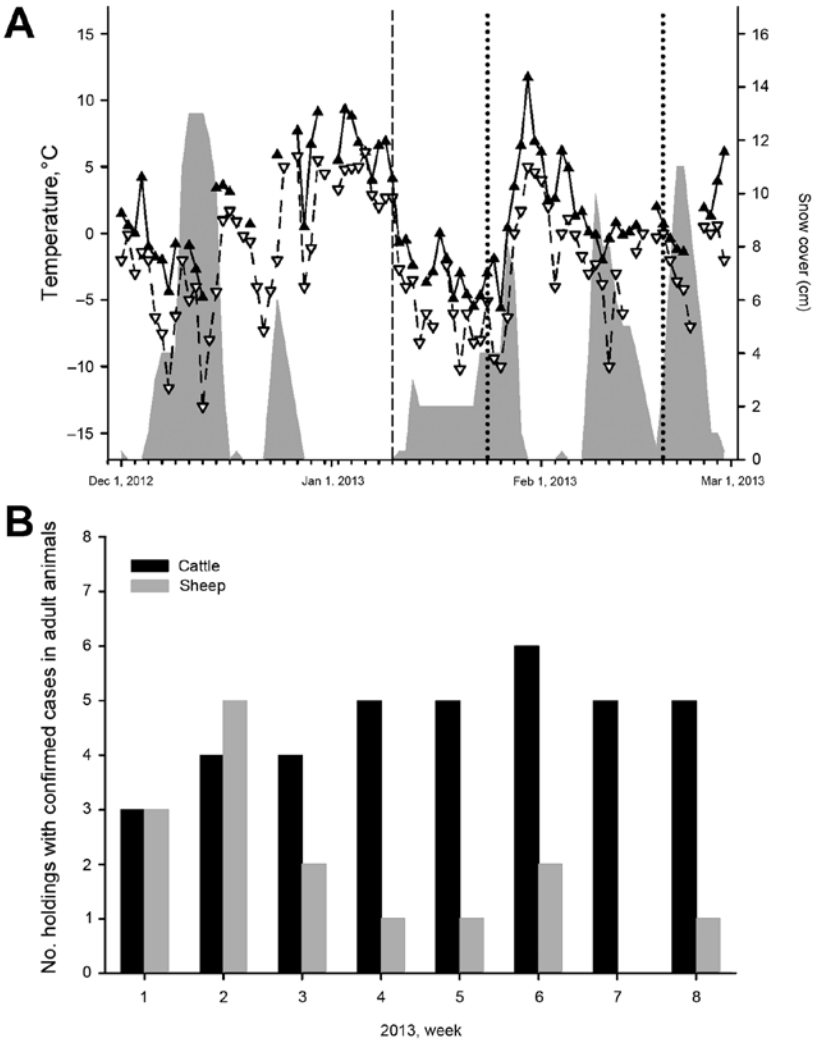

Figure. Results of analysis of samples from sheep and cattle for Schmallenberg virus (SBV), Germany, 2012–2013. A) Climate data and sampling. The maximum temperatures are shown with filled triangles and a solid line and the minimum temperatures with unfilled triangles and a broken line. Snow cover is symbolized by a gray area. The dashed line represents the day of the detection of SBV genome in 4 sheep. Further sampling days are marked by dotted lines. B) PCR-confirmed Schmallenberg virus infections in adult cattle (black bars) or sheep (gray bars) in Germany during January 1–February 20, 2013.

were seropositive. Because antibodies may be detectable 10 days–3 weeks after experimental infection for the first time (*8*), the presumed period of infection was between mid-January and mid-February. At this time, the highest temperatures again rose above 6°C for a few days (Figure 1, panel A).

Although the within-herd seroprevalence was >90% in ewes after confirmed or suspected SBV infection in 2011 (*9*), in this study, conducted during the cold season, only 12 (13%) of 90 tested sheep were positive by ELISA. Three animals seroconverted between mid-January and mid-February. Thus, SBV transmission appears to be possible at a low level, most likely because of the low activity of the involved insect vectors.

In addition to the SBV cases found on the sheep holding in Mecklenburg–Western Pomerania, an additional 52 confirmed SBV cases (defined as virus detection by qRT-PCR or isolation in cell culture) in adult ruminants were reported to the German Animal Disease Reporting System from January 1 through February 20, 2013 (Figure, panel B). Most affected animal holdings were located in Bavaria, but cases were also reported from Thuringia, Saxony, Brandenburg, Mecklenburg–Western Pomerania, Hesse, and Lower Saxony. In conclusion, transmission of SBV by hematophagous insects seems possible, even during the winter in central Europe, if minimum temperatures rise above a certain threshold for several consecutive days.

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Recurrent *Bordetella holmesii* Bacteremia and Nasal Carriage in a Patient Receiving Rituximab

To the Editor: We report a case of recurrent *Bordetella holmesii* bacteremia with 4 clinical manifestations: 3 episodes of cellulitis and 1 episode of pneumonia. The patient, a 67-year-old man, was admitted to the Pitié-Salpêtrière hospital in Paris, France, in December 2010, for recurrent cellulitis in his left leg. Eleven years earlier, diffuse large B-cell lymphoma had been diagnosed, and he had undergone 7 chemotherapy courses. He also had received 2 autologous stem cell transplants. He was receiving maintenance treatment with intravenous (IV) rituximab every 3 months and IV immunoglobulin for hypogammaglobulinemia.

The first episode of cellulitis had occurred in his left leg 2 months before admission; the condition was treated with pristinamycin (3 g/day for 14 days), and the leg healed completely. Cellulitis recurred in his left leg 2 months later; it was again treated with pristinamycin (3 g/day) for 4 days in conjunction with fusidic acid. The cutaneous lesions worsened, and

he was admitted to the hospital with fever (38.6°C) and chills.

Clinical examination showed extended cellulitis; the left leg was bright red, hot, shiny, swollen, and non-pitting. The patient's leukocyte count was 23 × 10^9/L (reference <10 × 10^9/L) and C-reactive protein level was 332 mg/L (reference <5 mg/L). IV clindamycin and ceftriaxone were administered. Fever and other symptoms improved rapidly. Two consecutive blood cultures carried out before antimicrobial drug treatment were positive for *B. holmesii*, according to biochemical characteristics and molecular detection of the specific *B. holmesii rec*A gene (*1*). Isolates in both cultures were susceptible to amoxicillin, macrolide antimicrobial drugs, cefoxitin, nalidixic acid, and ciprofloxacin and were resistant to cefotaxime and trimethoprim/sulfamethoxazole (Table; blood isolate, day 1). The antimicrobial drug regimen was changed to amoxicillin (6 g/day) for 14 days; the cellulitis resolved, and the patient was discharged.

Cellulitis in the right leg was diagnosed 2 weeks after the end of the previous treatment. Pristinamycin (3 g/day) was prescribed by the man's physician but was ineffective. He was readmitted, and *B. holmesii* was again isolated in 2 new blood cultures; the organism was now resistant to cefoxitin (Table; blood isolate, day 24). Oral amoxicillin was initiated (6 g/day), without success, and after 1 week, IV ceftriaxone (2 g/day) was administered. *B. holmesii* was again isolated (isolate blood, day 33) from blood cultures despite amoxicillin treatment, and the antibiogram had the same resistance profile, except for amoxicillin (which was not determined). Because the patient was improving, IV ceftriaxone was maintained for 18 days, and he was discharged 5 days after the beginning of efficient antimicrobial drug therapy.

Two weeks after the end of the treatment, the patient was admitted to the hospital for bilateral pneumonia. Treatment with piperacillin/tazobactam and ciprofloxacin for 14 days (750 mg 2×/day) was initiated. *B. holmesii* was again isolated from blood; the bacterium had now acquired resistance to amoxicillin and nalidixic acid (Table; isolate blood, day 74). Nevertheless, ciprofloxacin treatment was continued. By real-time PCR targeting of IS*481*, *Bordetella* DNA was detected in nasopharyngeal swab (NPS) specimens (*1*), but the species could not be identified because of an insufficient amount of DNA. One month after the end of the treatment, the patient was recovering. Although the patient was asymptomatic, *B. holmesii* was isolated in a second NPS specimen. The isolate was sensitive to amoxicillin and macrolides and resistant to cefotaxime, nalidixic acid, trimethoprim, and trimethoprim/sulfamethoxazole (Table; isolate NPS, day 105). Rituximab was discontinued, and relapse had not occurred after >1 year of follow-up.

B. holmesii was first described in 1995 (*2*); it was primarily isolated from the blood of immunocompromised patients, especially those with spleen dysfunction. Since 1999, *B. holmesii* has been detected during pertussis outbreaks in NPS specimens of patients with pertussis-like signs and symptoms (*3–6*). To our knowledge, the association between *B. holmesii* infection and rituximab treatment has been reported only once, in a renal transplant recipient, and *B. holmesii* nasal carriage was not tested for (*7*).

In this patient, the *B. holmesii* infection relapses definitively stopped after rituximab treatment was interrupted, which suggests a relationship between the 2 events and that patients receiving rituximab are at increased risk for severe infection (*8*). Interpretations of antimicrobial drug resistance are difficult because no breakpoints have been defined for this species, but MICs of the drugs showed changes in the resistance profile between infectious episodes (Table). These observations strongly suggest a heterogeneous population of bacteria and that resistance was acquired after antimicrobial drug treatment as described in the United Kingdom (*9*). The patient improved while receiving ceftriaxone, although, in vitro; the bacterium was found resistant to this antimicrobial drug family as reported (*10*). Thus, the in vitro susceptibility testing and in vivo efficacy were discordant.

In conclusion, the patient's nasal carriage and rituximab treatment may explain the recurrent infection. That the nasal carriage was the primary mode of transmission could not be proven because NPS specimens were not taken early enough. More studies

Table. Antimicrobial resistance profile of *Bordetella holmesii* isolates in vitro, France,, December 2010–March 2011*

	Antimicrobial drug MICs†, μg/mL, by isolate‡				
Antimicrobial agent	Blood, d 1	Blood, d 24	Blood, d 33	Blood, d 74	NPS, d 105
Amoxicillin	<2	<2	NA#	**16**	<2
Cefoxitin	<8	**>256**	**>256**	**>256**	**>256**
Cefotaxime	**>32**	**>32**	**>32**	**>32**	**>32**
Nalidixic acid	<16	<16	<16	**64**	**>256**
Trimethroprim	**>32**	**>32**	**>32**	**>32**	**>32**
Sulfamethoxazole	**>512**	**>512**	**>512**	**>512**	**>512**

*MICs corresponding to a drug resistance, which may reflect the general interpretation for nonfermenting bacteria, are in **boldface.** NPS, nasopharyngeal swab; NA, no available data.
†MICs were determined by E-test on Bordet–Gengou agar.
‡Site and day (d) of collection of isolate.

are needed to determine the role of nasal carriage in *B. holmesii* bacteremia. That no *B. holmesii* infections occurred after rituximab was stopped suggests that rituximab played a role in the recurrent infections. In cases of recurrent infection or bacteremia, nasal carriage should be assessed, and the interruption of rituximab should be considered by physicians.

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Rickettsia africae in *Amblyomma variegatum* Ticks, Uganda and Nigeria

To the Editor: *Rickettsia africae* is the most widespread spotted fever group (SFG) rickettsia in sub-Saharan Africa, where it causes African tick-bite fever (*1*), an acute, influenza-like syndrome. The number of cases in tourists returning from safari in sub-Saharan Africa is increasing (*1*). In western, central, and eastern sub-Saharan Africa, *R. africae* is carried by *Amblyomma variegatum* (Fabricius, 1794) ticks (*2*); usually associated with cattle, this 3-host tick also can feed on a variety of hosts, including humans (*2*). *R. africae* has not been reported in Uganda and rarely reported in Nigeria (*3*,*4*). Our objective was to determine the potential risk for human infection by screening for rickettsial DNA in *A. variegatum* ticks from cattle in Uganda and Nigeria.

In February 2010, ticks were collected from zebu cattle (*Bos indicus*) from 8 villages in the districts of Kaberamaido (Adektar [1°81′N–33°22′ E], Awimon [1°66′N–33°04′ E], Kalobo [1°88′ N–33°25′ E], Odidip [1°90′ N–33°30′ E], Odikara [1°91′ N–33°30′ E], and Olilimo [1°75′ N–33°38′ E], and Dokolo (Alela [2°09′ N–33°16′ E], and Angeta [1°87′ N–33°10′ E]) in Uganda and, in June 2010, in 3 villages (Mangar [9°14′ N–8°93′ E], Ruff [9°43′ N–9°10′ E], and Tambes [9°38′ N–9°38′ E]) in the Plateau State in Nigeria (Figure). This convenience sample was obtained as part of other ongoing research projects in both countries. Ticks were preserved in 70% ethanol and identified morphologically to the species level by using taxonomic keys (*5*). Because the anatomic features do not enable an objective assessment of the feeding status of adult male ticks, engorgement level was determined only in female tick specimens and nymphs.

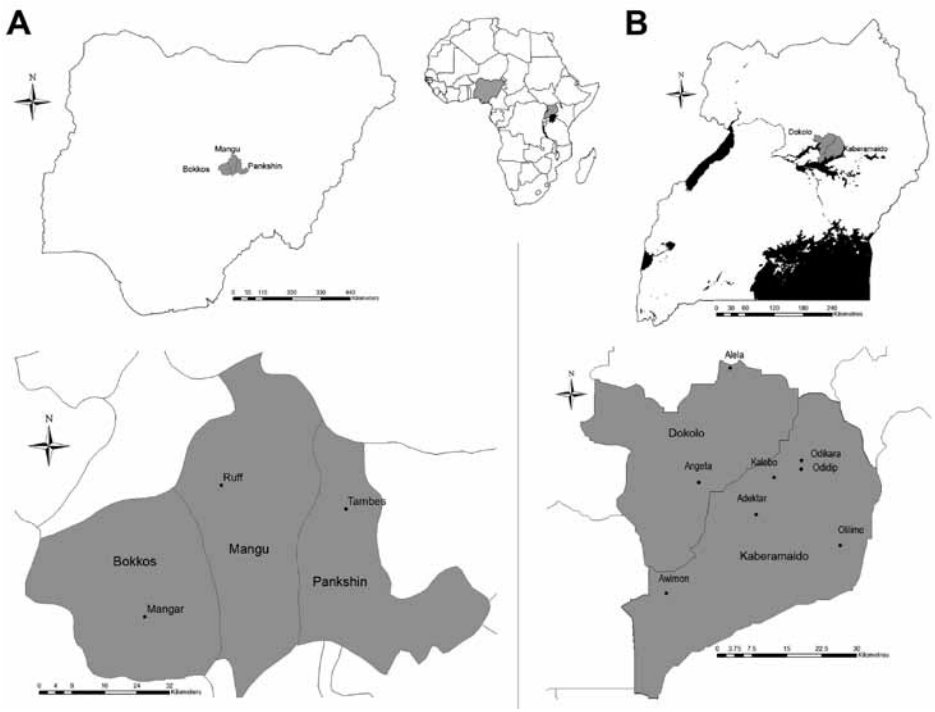

Figure. Location of areas studied for *Rickettsia africae* in *Amblyomma variegatum* ticks in Nigeria (A) and Uganda (B), 2010.

After tick identification, DNA was extracted from ticks by using QIAmp DNeasy kits (QIAGEN, Hilden, Germany). Two PCR targets were assessed within each sample; the primer pair Rp.CS.877p and RpCS.1258n was selective for a 396-bp fragment of a highly conserved gene encoding the citrate synthase (*glt*A) shared by all *Rickettsia* spp. (*6*); the Rr190–70p and Rr190–701n primer pair amplified a 629–632-bp fragment of the gene encoding the 190-kD antigenic outer membrane protein A (*omp*A), common to all SFG rickettsiae (*6*,*7*). DNA extracted from 2 *A. variegatum* tick cell lines (AVL/CTVM13 and AVL/CTVM17), previously amplified and sequenced by using primers for *Rickettsia* 16S rRNA, *omp*B, and *sca4* genes revealing >98% similarity with *R. africae* (*8*), was used as a positive control. Negative controls consisted of DNA from 2 male and female laboratory-reared *Rhipicephalus appendiculatus* ticks and distilled water. DNA of positive samples was recovered, and confirmation of amplicon authenticity was obtained through sequence analysis by using nucleotide BLAST (www.ncbi.nlm.nih.gov/BLAST).

A total of 39 ticks were collected in Uganda (32 adult males, 5 females, and 2 nymphs), and 141 were collected in Nigeria (80 males, 59 females, and 2 nymphs); all were identified as *A. variegatum* (online Technical Appendix Table, wwwnc.cdc.gov/EID/articlepdfs/19/10/13-0389-Techapp1.pdf). SFG rickettsiae DNA was amplified in 26 (67%) of 39 ticks from Uganda and 88 (62%) of 141 ticks from Nigeria by using the *omp*A gene primers; amplicons of the *glt*A genes were obtained in 16 (41%) of 39 ticks and 84 (60%) of 141 ticks, respectively (online Technical Appendix Table). Overall, 81 (45%) of 180 ticks were positive by *glt*A and *omp*A PCRs (online Technical Appendix Table). DNA sequences of the 22 *glt*A and *omp*A products from Uganda and the 22 from Nigeria showed 100% similarity with published sequences of *R. africae* (GenBank accession nos. U59733 and RAU43790, respectively). For both countries, ticks positive for *Rickettsia* spp. and SFG rickettsiae DNA were male and female specimens (online Technical Appendix Table). Among females, both unengorged and engorged specimens contained DNA from rickettsiae and SFG rickettsiae (online Technical Appendix Table).

These findings represent a novelty for Uganda. With reference to Nigeria, our results contrast with the prevalence of 8% recorded in a similarly sized sample (n = 153) of *A. variegatum* ticks collected from cattle in the same part of the country (*3*); this discrepancy might be the result of previous targeting of the rickettsial 16S rDNA gene. In the study reported here, the SFG-specific *omp*A PCR proved to be more sensitive than *glt*A for detecting rickettsiae DNA, as has also been reported in previous work (*9*). Although finding *R. africae* DNA in engorged female and nymphal tick specimens might be attributable to prolonged rickettsemia in cattle (*10*), the presence of *R. africae* in distinctly unengorged female ticks indicates the potential for *A. variegatum* ticks to act as a reservoir of this SFG rickettsia (*2*).

This study extends the known geographic range of *R. africae* in *A. variegatum* ticks in sub-Saharan Africa. The number of potentially infective ticks recorded in Uganda and Nigeria suggests that persons in rural areas of northern Uganda and central Nigeria might be at risk for African tick-bite fever. Awareness of this rickettsiosis should be raised, particularly among persons who handle cattle (e.g., herders and paraveterinary and veterinary personnel). Physicians in these areas as well as those who care for returning travelers, should consider African tick-bite fever in their differential diagnosis for patients with malaria and influenza-like illnesses.

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Ongoing Measles Outbreak in Orthodox Jewish Community, London, UK

To the Editor: Measles outbreaks have been reported in Orthodox and ultra-Orthodox Jewish communities across Europe and Israel (*1*–*5*). We describe an ongoing outbreak within the largest European Orthodox Jewish community (including a Charedi population of 17,587), based in London, focused in Hackney (*6*). Vaccination coverage within this community is lower than in the general population of London, causing low herd immunity and outbreaks of vaccine-preventable diseases. Vaccination coverage data within the communities cannot be extrapolated, because membership is not classified as an ethnicity and not collected within health electronic recording systems. However, general practice surgeries in Hackney known to have high proportions of Orthodox Jewish patients have considerably lower vaccination coverage (55%–75% of patients 24 months of age had received measles, mumps, rubella [MMR] vaccine in the 3rd quarter of 2012) compared with the London average (87.3%) (*7*). Health beliefs, family size (the average Charedi household size is 6.3 persons), and underutilization of immunization services contribute to low coverage (*8*,*9*).

The outbreak clinical case definition was taken from Public Health England's guidance (*10*). It also included membership in the Orthodox

Jewish community; residency in the London boroughs of Barnet, Hackney, or Haringey; and notification during December 20, 2012–March 19, 2013.

After serologic confirmation of measles in the index case-patient, an unvaccinated Orthodox Jewish 4-year-old from Hackney, the case was reported to the Health Protection Team (HPT) on December 20, 2012. The family could not recall having contact with someone with measles. The child attended nursery while infectious; subsequently, cases in 3 secondary patients in the nursery were reported to the HPT. Transmission was observed within households, extended family groups, nurseries, schools, and a camp for Orthodox Jewish teenagers attended by 80 girls (mainly from Hackney) with staff from Italy. Five secondary cases from this camp were reported (in 3 residents of London, 1 resident of Sheffield, and 1 resident of Hertfordshire).

During December 20, 2012–March 19, 2013, a total of 62 notifications of measles cases meeting the case definition were received in residents of Barnet (8 cases), Hackney (47), and Haringey (7). Patients' ages ranged from 7 months to 27 years (median 7 years). Thirty-four (55%) were female. Fifty-four (87%) had never received an MMR vaccine, and 8 (13%) had received only 1. Three were admitted to the hospital, and 5 were clinically assessed in accident and emergency departments (patients' ages ranged from 7 months to 4 years).

All case-patients were assessed for risk by the local HPT for vulnerable contacts and source of infection. The HPT provided infection control guidance and an oral fluid testing kit. Sixteen (26%) case-patients could not recall any contact with a person with measles; the remainder stated various epidemiologic links to a case-patient (Figure).

Forty-two cases have been confirmed (measles IgM detected) by serologic testing (4 cases) or oral fluid (38). One notified case-patient did not have measles IgM on oral fluid testing but had an epidemiologic link to a case-patient and clinical symptoms. They are included in this analysis. Seventeen IgM-positive oral fluid samples were genotyped, and all were D8, currently the most common genotype in the United Kingdom.

One confirmed case was detected in an unvaccinated child from Haringey who was not Orthodox Jewish but was known to have had contact with a case-patient from the community. The child's illness did not meet the case definition and is not included in this analysis.

In response to the outbreak, active case finding and awareness-raising have been undertaken by the HPT, National Health Service (NHS) public health departments, and community NHS services focused on health and education services and Orthodox Jewish communities. Information letters were sent to the 38 Orthodox Jewish schools and nurseries in Hackney and to attendees of the youth camp. Community NHS vaccination clinics have been maintained to complement standard immunization services offered in general practice surgeries. This includes a Sunday vaccination clinic. Furthermore, community NHS staff provided a vaccination clinic in a secondary school that had an attack rate of 7% (9 cases) at which 9 pupils received 1 MMR vaccine after parental consent. This was the only on-site school vaccination clinic offered; thus,

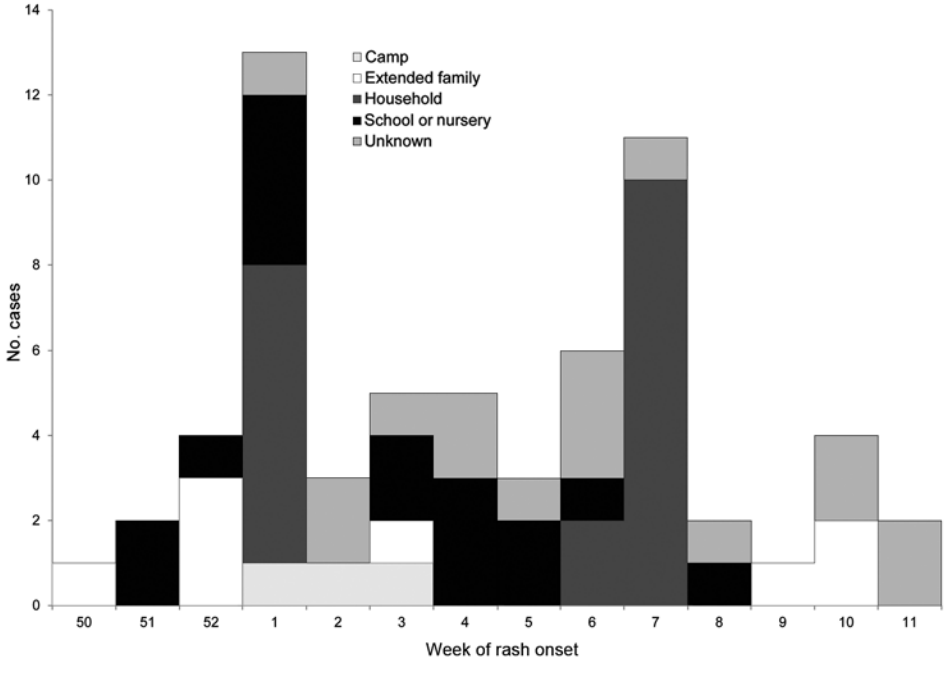

Figure. Reported measles cases by week of rash onset and likely source of infection, United Kingdom, 2012–2013.

no comparative uptake data are available to supplement our evaluation of the intervention.

Information relating to the outbreak was placed in 2 Orthodox Jewish newspapers and targeted information for families (in English, Yiddish, and Hebrew) has been disseminated. Finally, all 25 HPTs were alerted to this outbreak and the national Public Health England database (HPZone) has been enhanced to capture notifications from Orthodox Jewish communities.

This ongoing outbreak highlights continued health risks in communities with low vaccination coverage. The outbreak has been largely contained within London's Orthodox Jewish communities, with limited spread outside of the city and to just 1 local non–Orthodox Jewish child. Given the mobility of members, the risk for transmission outside of London is relatively high. The outbreak underscores the need for ongoing evidence-based and culturally appropriate health interventions that seek to improve vaccination coverage.

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Human Infection with Eurasian Avian-like Influenza A(H1N1) Virus, China

To the Editor: We report a human infection with avian-like swine A(H1N1) influenza virus first identified through a surveillance system for influenza like illness (ILI) in mainland China. An influenza virus, isolated from a patient with ILI, was originally subtyped as influenza A(H1N1)pdm09 virus with a hemagglutination inhibition (HI) test, but it was identified as a Eurasian avian-like influenza A(H1N1) virus (EA-H1N1) by full genome sequencing on January 30, 2013. The virus was named A/Hebei-Yuhua/SWL1250/2012 (H1N1v) (HB/1250/12), according to the definition of the World Health Organization (*1*).

The case-patient was a 3-year-old boy who had symptoms of fever and sore throat; his highest body temperature was 38°C on December 9, 2012. He was brought for medical treatment to an influenza sentinel hospital in the city of Shijiazhuang in Hebei Province, China, on December 12. He recovered within a week without hospitalization and oseltamivir treatment. A throat swab specimen was collected and sent to the local Chinese Center for Disease Control and Prevention for virus isolation and characterization, according to the Guidelines of the Chinese National Influenza Surveillance Network. A retrospective investigation was conducted to identify the potential infection source and any other possible cases. The case-patient was previously healthy and had no history of close contact with animals (live or dead wild birds, poultry, and swine) within 2 weeks before the onset of symptoms, nor a history of travel. He lived with his sister and parents; all other family members did not

develop influenza-like symptoms during the period of the investigation.

Sporadic human infections with swine influenza virus had been reported previously (*2*,*3*). Another case-patient, infected by EA-H1N1 influenza virus A/Jiangsu/1/2011(JS11) in early 2011, was reported (*4*,*5*). The genome sequences of the viruses isolated from the 2 case-patients showed high homology; the similarity of the polymerase basic protein 2 was 99.1%; of polymerase basic protein 1, 99.3%; of polymerase acidic protein, 98.9%; of hemagglutinin (HA), 99.1%; nucleocapsid protein, 99.1%; neuraminidase protein, 99.2%; matrix protein, 99.6% and of nonstructural protein, 99.2% (Global Initiative on Sharing Avian Influenza Data, GISAID, accession no.EPI301156–63 for JS11 and EPI438417–25 for HB/1250/12). The HB/1250/12 virus has the amino acids D (at site 190) and E (at site 225) within the HA protein, which are reported to be critical for enhancement of the HA affinity in binding to a-2,6–linked sialosides (*6*). The virus was resistant to amantadine and rimantadine with S31N (Ser→Asn) mutation in M2 gene, but was predicted to be susceptible to the neuraminidase inhibitor drugs oseltamivir and zanamivir on the basis of the neuraminidase gene.

HI test with ferret anti-serum against A(H1N1)pdm09 (CA09), seasonal H3N2(Vic11,BR10/07 and Perth09), classical swine subtype H1N1(NJ76), and the seasonal influenza subtype H1N1 viruses (BR59/07, SI06) showed that the HB/1250/12 virus is antigenically indistinguishable from NJ76 and CA09, but different from subtype H3N2 viruses (Vic11, BR10/07, and Perth09) and seasonal subtype H1N1 viruses(BR59/07, SI06,) (online Technical Appendix Table; wwwnc.cdc.gov/EID/article/19/10/13-0420-Techapp1.pdf). These findings were consistent with results reported previously (*7*–*9*).

To estimate the susceptibility of human population to this virus, and to investigate whether seasonal trivalent inactivated influenza vaccine (TIV) could provide cross-protection, we collected serum samples from children, adults, and elderly adults, before and after 2012–2013 TIV vaccination, and the antibody against HB/1250/12 virus was tested by HI assay. The seroprotection antibody was defined as HI titers ≥40. Before vaccination, 28% of children (3–5 years) and 6.7% of adults (18–59 years) had HI titers ≥40, but elderly adults (>60 years) did not. Samples from 56% of children, 56.7% of adults, and 26.7% of elderly adults had HI titers ≥40 after TIV vaccination; however, a 4-fold antibody rise developed in <30% in any age group (Table). These results indicated that a proportion of cross-protective antibody against EA-H1N1 exists in children and adults, whereas elderly adults are the most susceptible to EA-H1N1 infection with no cross-protective antibody, the vaccination with TIV could not substantially improve the level of cross-reactive EA-H1N1 antibodies.

Antisera from hyperimmune sheep are usually used for influenza virus typing and subtyping, the CA09 sheep antisera reacted well with the HB/1250/12 virus (online Technical Appendix Table). This is the reason why the local Chinese Center for Disease Control and Prevention originally subtyped HB/1250/12 as A(H1N1) pdm09 virus. Such avian-like H1N1 virus could be missed with regular HI test. In addition, a large proportion of swine influenza infection cases are mild and even asymptomatic (*2*); thus, the human infections with swine influenza virus may have been underestimated in China.

This is the first human case of EA-H1N1 infection identified through the national ILI surveillance network in China, indicating that the influenza surveillance network not only plays a critical role in monitoring the seasonal influenza circulation and the vaccine virus selection, but also is useful for early detection of novel influenza viruses with pandemic potential. This study also highlighted the value of, and urgent demand for, a cost-effective sequencing platform on routine influenza surveillance for pandemic preparedness.

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Table. Cross-reactive antibody response against avian-like influenza A(H1N1) virus in pediatric and adult recipients of seasonal trivalent inactivated influenza vaccines, China, 2013*†

Age group, y	Antigen	Increase ≥4, %‡	Geometric mean titer		%Titer ≥40		%Titer ≥160	
			Before vac.	After vac.	Before vac.	After vac.	Before vac.	After vac.
Children, 3–5, n = 25	A/California/7/2009	60.0	21.1	121.3	44.0	84.0	16.0	60.0
	HB/1250/12	24.0	12.8	25.7	28.0	56.0	0	24.0
Adults, 18–59, n = 30	A/California/7/2009	70.0	14.8	156.3	26.7	76.7	0	60.0
	HB/1250/12	6.7	8.1	31.7	6.7	56.7	0	23.3
Elderly adults, ≥60, n = 30	A/California/7/2009	46.7	10.5	52.8	10.0	53.3	0	30.0
	HB/1250/12	26.7	5.7	11.5	0	26.7	0	6.7

*Vac., vaccination.
†All children received 2 doses of vaccine with an interval of 1 month. The composition of the trivalent vaccine were A/Christchurch/16/2010(NIB-74xp) (A/California/7/2009-like), A/Victoria/361/2011 (H3N2)IVR-165, and B/Hubei-Wujiagang/158/2009. Serum samples were obtained from vaccine recipients living in northern (children) and southern (adults and elderly adults) China.
‡Increase in antibody titer.

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Novel Bat Coronaviruses, Brazil and Mexico

To the Editor: Bats are now recognized as natural reservoirs for many families of viruses that can cross species barriers and cause emerging diseases of humans and animals. Protecting humans against emerging diseases relies on identifying natural reservoirs for such viruses and surveillance for host-jumping events. The emergence of the Middle East respiratory syndrome coronavirus (MERS-CoV) on the Arabian Peninsula (*1*) further justifies increased surveillance for coronaviruses (CoVs) in bats. MERS-CoV most likely is a zoonotic virus from a bat reservoir and is associated with high case-fatality rates among humans. The existence of a diverse array of alphacoronaviruses in bats in the United States, Canada, and Trinidad has been reported (*2–6*). Recently, a possible new alphacoronavirus was detected in an urban roost of bats in southern Brazil (*7*), and a survey of bats in southern Mexico reported 8 novel alphacoronaviruses and 4 novel betacoronaviruses, 1 with 96% similarity to MERS-CoV (*8*). These findings expand the diversity and range of known bat coronaviruses and increase the known reservoir for potential emerging zoonotic CoVs.

Expanding on our previous work (*2*,*3*), we analyzed samples from 97 bats from Brazil and 75 bats from Mexico (Technical Appendix, wwwnc.cdc.gov/EID/article/19/10/13-0525-Techapp1.pdf). During 2007–2010, intestinal samples were collected from bats of 10 species in northwest São Paulo state in southeastern Brazil. These bats had been submitted to the University Estadual Paulista for rabies testing as a result of epidemiologic surveillance or, in some cases, because of possible or known contact with humans. During 2011–2012, as part of an ongoing rabies surveillance project, intestinal samples were collected from bats of 12 species in their usual habitats in Jalisco state in midwestern Mexico. Bats from a variety of species, including insectivorous, nectarivorous, frugivorous, and hematophagous bats, were included in this study for the purpose of obtaining a diverse array of potential exposures. Intestines were collected and stored, and RNA was purified as described (*2*). CoV RNA was detected by using a pancoronavirus PCR selective for the RNA-dependent RNA polymerase gene, and amplicons were sequenced as described (*3*). Virus isolation was not attempted as part of this study.

From 1 of 17 *Molossus rufus* bats and 1 of 8 *Molossus molossus* bats, an identical novel alphacoronavirus was detected (BatCoV-*M.rufus*28/Brazil/2010, GenBank accession no. KC886321). Both specimens were collected in Brazil during 2010 from adult male bats that had been found in urban areas on residential property. The 412-nt sequence of this virus was most closely related to alphacoronaviruses detected in *Eptesicus fuscus* bats in North America (82% nt

identity), *Myotis australis* bats in Australia (77% nt identity), *Miniopterus* bats in Kenya (77% nt identity), and *Rhinolophus* bats in Hong Kong (77% nt identity) (Figure). Bats of the genus *Molossus* are insectivorous; their geographic range is restricted to the New World, from northern Mexico to northern Argentina.

A novel betacoronavirus (presumably group C) was detected in a specimen from 1 of 4 *Pteronotus davyi* bats (BatCoV-*P.davyi*49/Mexico/2012, GenBank accession no. KC886322). This specimen was collected in 2012 from an adult male bat roosting in a cave in La Huerta, Mexico. The 439-nt sequence of this virus has 71% nt identity to the novel human group C betacoronavirus MERS-CoV and 72% nt identity to various group D betacoronaviruses detected in *Rousettus*, *Pipistrellus*, and *Tylonycteris* bats in the Philippines, China, and Kenya (Figure). Bats of the species *P. davyi* (Davy's naked-backed bat) are insectivorous and are found from southern Mexico to the northern parts of South America. They prefer to roost in caves and man-made structures, such as mines.

In summary, we found a novel alphacoronavirus in bats from Brazil and a novel betacoronavirus in a bat from Mexico. Both viruses were detected in bats with known or potential contact with humans. Because the bats we sampled were mostly adult males, the prevalence of CoVs that we identified is probably an underestimation of the true incidence of CoVs in these bat populations. For bats of other species, incidence of CoVs among juvenile and female bats is higher (*2*,*9*). Furthermore, we used a non-nested, broadly conserved CoV PCR, which might have limited the sensitivity of CoV RNA detection. The finding of a novel betacoronavirus in insectivorous bats in the New World is noteworthy. Three human CoVs (229E, SARS-CoV, and MERS-CoV) all have animal reservoirs of closely related viruses in Old World insectivorous bats (*10*) from which they most likely emerged, either directly or indirectly, into the human population. Ongoing surveillance for CoVs in wildlife and increased research efforts to better understand the factors associated with CoV host-switching events are warranted.

Figure. Phylogenetic tree showing relationships based on 412-nt and 439-nt sequences of a conserved region of gene 1b of BatCoV/Molossus rufus28/Brazil/2010 (alphacoronavirus) and BatCoV/Pteronotus davyi49/Mexico/2012 (betacoronavirus) to other known coronaviruses. Sequences were aligned by using ClustalW (www.clustal.org/), phylogenetic analyses were conducted by using the neighbor-joining method and BioEdit (www.mbio.ncsu.edu/BioEdit/BioEdit.html), and trees were constructed by using FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Boxes surround the novel alphacoronavirus detected in *Molossus rufus* and *M. molossus* bat specimens from São Paulo state in southeastern Brazil, and the novel betacoronavirus detected in a specimen from a *Pteronotus davyi* bat from Jalisco state in midwestern Mexico. GenBank accession numbers aree AB539081.1, DQ648808.1, GU065420.1, HM211099.1, EF065512.1, EF065512.1, JX869059.2, GU065398.1, EF507794.1, FJ710054.1, JX537914.1, EF544566.1, EU834956.1, EF203064.1, GU065410.1, HQ728484.1, GU065409.1, HQ184049.1, DQ666339.1, HQ336974.1, DQ445911.1, KC210147.1, AY278741, NC_002645.1, and NC_005147.1. PEDV, porcine epidemic diarrhea virus. Scale bar indicates nucleotide substitutions per site.

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Vibrio cholerae O1 El Tor and O139 Bengal Strains Carrying *ctxB*^{ET}, Bangladesh

To the Editor: Cholera, caused by *Vibrio cholerae*, continues to affect millions of persons in disease-endemic areas where safe drinking water is scarce and sanitation is poor. Of 7 cholera pandemics recorded since 1817, *V. cholerae* serogroup O1 classical (CL) biotype was associated with the sixth, whereas the seventh (ongoing) pandemic was initiated by *V. cholerae* O1 biotype El Tor (ET), which displaced CL in the early 1960s (*1*). During 1992–1993, a *V. cholerae* non-O1 serogroup, designated *V. cholerae* O139 synonym Bengal, initiated cholera epidemics in India and Bangladesh by transiently displacing *V. cholerae* O1 ET biotype (*2*). *V. cholerae* O139 was less frequently associated with cholera in Bangladesh than *V. cholerae* ET in 1994 and the years following, until 2005 (*3*); it has been undetected since then. Meanwhile, *V. cholerae* ET has shown genetic changes since 2001, and isolates carry the *ctxB* gene of the CL biotype (*ctxB*^{CL}) in Bangladesh (*4*). Although the genetic transition from *ctxB*^{ET} to *ctxB*^{CL} was observed during 1998–1999 for *V. cholerae* O139 (*5*), *V. cholerae* strains carrying *ctxB*^{ET} were considered extinct, i.e., undetected for about a decade.

During June 2010–December 2012, the International Centre for Diarrheal Disease Research, Bangladesh (ICDDR,B) systematically conducted ongoing epidemiologic ecologic surveillance in Dhaka, Chhatak, and Mathbaria and isolated *V. cholerae* strains (n = 500 [clinical/environmental]: Dhaka [n = 110/94], Mathbaria [n = 90/79], Chhatak [n = 111/16]). Of the 500 *V. cholerae* isolates, 496 were confirmed as O1 and 4 as O139 Bengal, on the basis of serologic, phenotypic, and genetic properties (*3*,*6*–*8*).

All *V. cholerae* O1 and O139 isolates were positive for *ctxA*, *tlc*, *ace*, and *zot* and possessed ET biotype–specific markers *tcpA*^{ET}, *hlyA*^{ET}, and *rtxC*. Mismatch amplification mutation assay–PCR (*9*) demonstrated *ctxB*^{CL} allele in 492 *V. cholerae* O1 ET strains (altered ET), whereas *ctxB*^{ET} was found in 8 isolates (4 *V. cholerae* O1 ET and 4 *V. cholerae* O139).

Nucleotide sequencing of *ctxB* showed that the translated sequences of *V. cholerae* O1 and O139 strains carrying *ctxB*^{ET} were identical to those of the ET reference strain N16961 (GenBank accession no. NC_002505), with tyrosine and isoleucine at positions 39 and 68, respectively, as opposed to altered ET, which possesses histidine and threonine at positions 39 and 68, respectively (*4*). PCR additionally showed that the *V. cholerae* O1 and O139 Bengal strains carrying *ctxB*^{ET} had the ET biotype–specific RS1 element gene *rstC* and repressor gene *rstR*^{ET}, suggesting prototype ET attributes (*7*).

Three *V. cholerae* strains carrying *ctxB*^{ET} were first isolated in 2011 from surface water: one O1 strain and one O139 strain from Mathbaria and one O1 strain from Chhatak. In 2012, *V. cholerae* O1 carrying *ctxB*^{ET} was isolated from cholera patients in Mathbaria and Chhatak (n = 1 each). Also, 3 O139 strains carrying *ctxB*^{ET} were isolated from surface water in Dhaka. The confirmed *V. cholerae* O1 and O139 Bengal strains carrying *ctxB*^{ET} were of particular interest because altered ET strains carrying *ctxB*^{CL} have been deemed the cause of endemic cholera in Bangladesh since 2001 (*4*) and globally (*10*).

V. cholerae strains carrying *ctxB*^{ET} were closely related to the pre-2001 *V. cholerae* strains carrying *ctxB*^{ET}, as were the O139 Bengal strains carrying *ctxB*^{ET}. Two lines of evidence support this close relationship. First, the antimicrobial drug resistance patterns of 3 of the *V. cholerae* O139 strains isolated in Dhaka during 2012 were resistant to trimethoprim/sulfamethoxazole (25 μg), whereas the remaining O139 and 4 O1 strains were susceptible to all drugs tested, including azithromycin (15 μg), ciprofloxacin (5 μg), gentamicin (10 μg), ampicillin (10 μg), tetracycline (30 μg), and erythromycin (15 μg). Second, pulsed-field gel electrophoresis (PFGE) of *Not*I-digested genomic DNA showed identical banding patterns for the 4 *V. cholerae* O1 strains carrying *ctxB*^{ET} and the pre-2001 ET strains, including N16961, and the DNA pattern differed from that of the altered ET associated with endemic cholera in Bangladesh (Figure). All 4 *V. cholerae* O139 strains had typical O139 Bengal banding patterns, shown by PFGE, except that 1 strain had an extra band (Figure). Comparison of PFGE patterns with those of previously isolated *V. cholerae* O139 strains (1993–2005) showed that recently isolated strains (2011–2012) belonged to 1 of the ancient clones, suggesting that the strain has been present in Bangladesh since 1993 (Figure).

In conclusion, we provide evidence of the coexistence of *V. cholerae* O1 and O139 strains, which shows that strains carrying *ctxB*^{ET}, not isolated for approximately a decade in Bangladesh, have again been isolated (*3*). Although the epidemiologic importance of the observed genetic change in the *ctxB* is yet to be understood, the finding of *V. cholerae* strains carrying *ctxB*^{ET} in surface water of Bangladesh in 2011 and in association the following year with

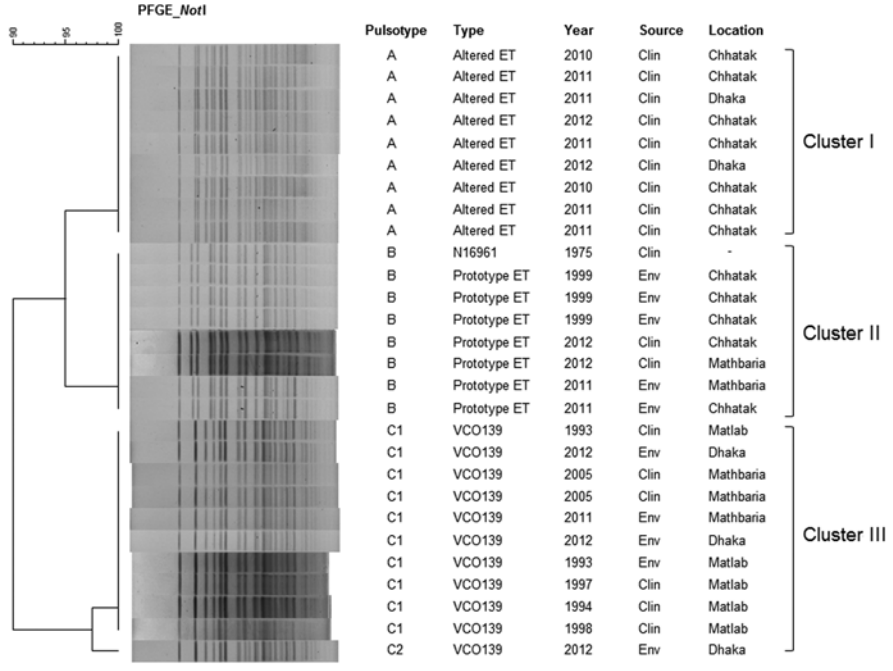

Figure. DNA fingerprinting patterns of *Vibrio cholerae*. Dendrogram was prepared by Dice similarity coefficient and UPGMA (unweighted pair-group method with arithmetic mean) clustering methods by using pulsed-field gel electrophoresis (PFGE) images of the *Not*I-digested genomic DNA. The scale bar at the top (left) indicates the correlation coefficient (range 90%–100%). *V. cholerae* altered ET (*ctxB*^{CL}) strains (pulsotype A) formed a major cluster (cluster I), separated from prototype ET (*ctxB*^{ET}) strains (cluster II; pulsotype B) and *V. cholerae* O139 strains (cluster III; pulsotype C), suggesting that they are genetically different. ET, El Tor; Clin, Clinical; Env, environmental.

cholera may be yet another turning point, considering that the global pattern of cholera is changing rapidly.

This study was supported in part by National Institute of Health grant no. RO1A1039129 (under collaborative agreements between the Johns Hopkins Bloomberg School of Public Health and ICDDR,B) and National Institute of Infectious Diseases, Japan. ICDDR,B acknowledges with gratitude the commitment of the National Institutes of Health and the National Institute of Infectious Diseases to its research efforts.

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Dust Bowl, 1933 (detail) Alexandre Hogue, 1898–1994 Oil on canvas 24 x 32 5/8 in. (61 x 82.8 cm) Smithsonian American Art Museum

The Ecology of Wrath

Polyxeni Potter

"Houses were shut tight, and cloth wedged around doors and windows, but the dust came in so thinly that it could not be seen in the air, and it settled like pollen on the chairs and tables, on the dishes," wrote John Steinbeck in The Grapes of Wrath. The same year, 1939, the author elaborated in a letter that his goal in writing the book was "to rip a reader's nerves to rags" by laying bare the life of the Dust Bowl migrants with whom he had spent time. Oklahoma Congressman Lyle Boren called the book "an infernal creation of a twisted distorted mind."

After Congress passed the Homestead Act in 1862, thousands of settlers moved to the semi-arid grasslands of the North American plains to farm and graze cattle. They plowed the fields and planted dryland wheat. High demand generated the promise of economic development and brought in more powerful plows, further expanding arable land. The grasses receded, leaving the ground exposed and vulnerable. When the drought came in 1930, strong winds whipping across the plains created severe dust storms, which continued for nearly a decade, moving millions of tons of topsoil and wiping out farms and ranches across 19 states in the heartland, which became known as the Dust Bowl.

"And then the dispossessed were drawn west–from Kansas, Oklahoma, Texas, New Mexico; from Nevada and Arkansas, families, tribes, dusted out, tractored out. Car-loads, caravans, homeless and hungry; twenty thousand and fifty thousand and a hundred thousand and two hundred thousand," Steinbeck wrote. Eleanor Roosevelt was less critical of his literary account, "The book is coarse in spots, but life is coarse in spots."

In 1898, Alexandre Hogue's family moved from Memphis, Missouri, where he was born, to Denton, Texas, where his life and art would later become inextricably connected with the Dust Bowl. His early copy of Millet's *The Gleaners*, a painting of peasant women gleaning a field of wheat after the harvest, foreshadowed Hogue's lifelong interest in rural society and the land. He attended the Minneapolis College of Art and Design and took lessons from Clarence Conaughy and the muralist Lauros Phoenix.

Hogue spent time in New York, absorbing the art scene and forming his own philosophy as painter, lithographer, printmaker, and muralist. "The true artist in painting or any other aesthetic expression sets out to express himself in terms of life he really knows." He denied lasting influence from any training or external source. "I was considered a dead-on radical when I was young."

After a 4-year stint in the big city, Hogue became a vocal member of the Dallas Nine and their circle, a group of artists active in the mid-1920s. Other well-known members were Jerry Bywaters, Otis Dozier, Olin Travis, William Lester, Everett Spruce, Thomas Stell, Perry Nichols, Harry Carnohan, and John Douglass. These artists believed in creating their own idiom by portraying the local scene.

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Beginning in 1926, Hogue spent time each year in New Mexico, until the beginning of World War II. There he met founding member of the Taos Society of Artists Ernest Blumenschein and American masters Joseph Imhof, Victor Higgins, Emil Bisttram, and Buck Dunton. He made sketching trips into Indian Territory and became acquainted with Indian culture. For creating lasting art, Hogue believed, a feeling for the locality was more important than the natural beauty of the area.

In the 1930s Hogue painted his Erosion series, depicting the devastation of the Dust Bowl, which he witnessed firsthand, "both before and after the dust menace, working and painting on a Panhandle ranch near Dalhart." It had been "Plowed in on all sides by the 'suitcase' farmers, whose uncontrolled loose dirt, pushed before the wind, has gnawed away every sprig of grass that dares show above ground." When the images appeared in national magazines, reaction was mixed. "Such misinformation will undoubtedly cause tourists and others to abandon or postpone visits to the many important and interesting points in the State of Texas."

Hogue described his work as "psycho-reality," involving "mind reactions to real situations, not dreams or subconscious." He converted his thoughts into abstract visual terms, which were stronger than nature itself. In his work *Drouth Stricken Area*, "The windmill and the drink tub are taken from life," he wrote. "I worked on that windmill. In fact I was knocked off it by lightning. It was the windmill that was on my sister and brother-in-law's place–the Bishop Ranch near Dalhart, Texas. The house was strictly my own. I just depicted it so it would be typical of the time…. The placing of a top of a shed coming in front of the tank is strictly a matter of composition. The whole thing is just visually built."

"Some may feel that in these paintings… I may have chosen an unpleasant subject, but after all the drouth *is* most unpleasant. To record its beautiful moments without its tragedy would be false indeed. At one and the same time the drouth is beautiful in its effects and terrifying in its results. The former shows peace on the surface but the latter reveals tragedy underneath. Tragedy as I have used it is simply visual psychology, which is beautiful in a terrifying way."

In the *Dust Bowl*, on this month's cover, the footprints and tire tracks leading away from the farm denote those who fled, the "exodusters." The angular shapes of fallen fence posts and tangled barbed wire mimic the blood-red wedge of thick dust against the sky. Along the rippled earth, a diminishing truck tire mark suggests that the family has just moved away, their traces already fading in the dust.

More people actually stayed on their land than left, and many died of malnutrition and dust pneumonia–not the only illness eventually associated with the dust. Many discoveries about coccidioidomycosis arose from careful epidemiologic and clinical investigations in California's San Joaquin Valley during the 1930s, when people migrated there from the Dust Bowl, and during the 1940s when World War II brought military recruits, prisoners of war, and persons of Japanese descent to camps and other areas where the disease is endemic.

Now reemerging, coccidioidomycosis is sometimes called San Joaquin Valley fever or Valley fever. It is caused by a soil fungus found in the southwestern United States, mostly California and Arizona, and in northern Mexico and parts of Central and South America. Dormant during long dry spells, it develops as a mold when the rains come. Spores are swept into the air when the soil is disrupted during construction, farming, earthquakes, or other dust-cloud generating events and are inhaled into the lungs, where the infection begins.

"I don't like to be called a 'regionalist' or 'American scene painter,' or, as *Life* magazine called me, 'painter of the Dust Bowl,'" proclaimed Hogue even as he urged farmers to cooperate with federal soil conservation efforts. "My paintings are as much a statement of what may happen as what has happened–a warning of impending danger in terms of present conditions."

Continued economic development changes the land and draws new populations into disease-endemic areas. In the past decade, state-specific increases in the number of reported coccidioidomycosis cases have been observed in Arizona and in California, where hospitalization costs are increasing. Hogue's ecologic concerns still apply because, as much as it is vital for creating lasting art, understanding the locality is indispensable for deciphering disease emergence, which is so much a circumstance of time and place. Steinbeck understood the complexity: "The land is so much more than its analysis."

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Article Title:

Increased Incidence of Invasive Fusariosis with Cutaneous Portal of Entry, Brazil

CME Questions

1. What was the most common underlying disease among patients with invasive fusariosis in the current study?

A. Multiple myeloma
B. Non-Hodgkin lymphoma
C. Acute myeloid leukemia
D. Chronic myeloid leukemia

2. Which of the following statements regarding the presentation of invasive fusariosis is most accurate?

A. Two-thirds of cases were notable for a cutaneous portal of entry of *Fusarium* spp.
B. All cases of cutaneous entry were associated with onychomycosis
C. Most cutaneous portals of entry were identified within 48 hours
D. Neutropenia was rare among cases of invasive fusariosis

3. Which of the following statements regarding the laboratory identification and management of invasive fusariosis in the current study is most accurate?

A. All patients had a positive blood culture
B. Fluconazole was the most common treatment used
C. The 30-day survival rate was 33.3%
D. Infecting *Fusarium* spp. were derived from the hospital's water system

4. What were the trends in the rates of invasive and cutaneous fusariosis between 2000 and 2010?

A. Increased incidence of both invasive and cutaneous fusariosis
B. Increased incidence of invasive fusariosis; reduced incidence of cutaneous fusariosis
C. Reduced incidence of invasive fusariosis; increased incidence of cutaneous fusariosis
D. Reduced incidence of both invasive and cutaneous fusariosis

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

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Article Title

Cryptococcus gattii infections in Multiple States Outside the US Pacific Northwest

CME Questions

1. You are asked to see a 40-year-old man with neurologic and pulmonary symptoms for the past several weeks. He had received treatment with azithromycin from an urgent care clinic, with no improvement. You consider whether this patient may have a fungal infection. Which of the following statements regarding *Cryptococcus gattii* infections is most accurate?

A. Transmission is primarily person-to-person
B. The incubation period is approximately 2 to 4 weeks
C. *C. gattii* primarily affects persons with HIV infection
D. There has been an outbreak of *C. gattii* in the Pacific Northwest United States

2. What should you consider regarding the presentation of patients with *C. gattii* infection in the current study?

A. Almost two-thirds of patients were healthy at the time of diagnosis
B. Half of patients were asymptomatic at the time of diagnosis
C. The average time from symptom onset to the diagnosis of *C. gattii* infection was 3 months
D. Cough and weight loss were the most common presenting symptoms

3. The patient is diagnosed with *C. gattii* infection of both the lungs and central nervous system. What should you consider regarding the management of patients with *C. gattii* infection in the current study?

A. Most patients did not require hospitalization
B. There were no admissions to the intensive care unit associated with *C. gattii* infection
C. Monotherapy with fluconazole was the most common treatment used
D. Over 35% of patients treated for *C. gattii* infection received corticosteroids

4. What should you consider regarding outcomes of patients with *C. gattii* infection in the current study?

A. The overall mortality rate exceeded 50%
B. All patients who died had underlying immunocompromised status
C. Nearly 40% of patients with follow-up data had cranial nerve palsies
D. Nearly all patients who died were men

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Transmissibility of Livestock-associated Methicillin-Resistant *Staphylococcus aureus*

Tropheryma whipplei Endocarditis

Mobile Phone–based Syndromic Surveillance System, Papua New Guinea

Pseudorabies Virus Variant in Pigs Immunized with Bartha-K61 Vaccine Strain, China

Influenza Virus Migration and Persistence, Vietnam, 2001–2008

Common Epidemiology of *Rickettsia felis* Infection and Malaria, Africa

Severe Influenza-associated Respiratory Infection in High HIV Prevalence Setting, South Africa, 2009–2011

Severe Fever with Thrombocytopenia Syndrome, South Korea, 2012.

Full Genome of Influenza A(H7N9) Virus Derived by Direct Sequencing without Culture

Incidence of Influenza A(H1N1)pdm09 Infection, United Kingdom, 2009–2011

Nontoxigenic Highly Pathogenic Clone of *Corynebacterium diphtheriae*, Poland, 2004–2012

Increased Incidence of *Campylobacter* spp. Infection and High Rates among Children, Israel

Middle East Respiratory Syndrome Coronavirus in Bats, Saudi Arabia

Mild Illness in Avian Influenza A(H7N9) Virus–Infected Poultry Worker, Huzhou, China, April 2013

West Nile Virus, Texas, USA, 2012

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

Upcoming Infectious Disease Activities

October 2–6, 2013
A combined meeting of the Infectious Diseases Society of America (IDSA),the Society for Healthcare Epidemiology of America (SHEA), the HIV Medicine Association (HIVMA) and the Pediatric Infectious Diseases Society (PIDS)
San Francisco, CA, USA
http://idweek.org

November 2–6, 2013
APHA
American Public Health Association's 141st Annual Meeting and Exposition
Boston, MA, USA
http://www.apha.org

November 4–7, 2013
3rd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals: Veterinary and Public Health Implications
Copenhagen, Denmark
http://www.asm.org/conferences

November 5–7, 2013
ESCAIDE 2013
European Scientific Conference on Applied Infectious Disease Epidemiology
Stockholm, Sweden
http://www.escaide.eu

November 13-17, 2013
ASTMH – American Society of Tropical Medicine and Hygiene Annual Meeting
Washington, DC
https://www.astmh.org/source/Registration/Main/MtgInfo.cfm?

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Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

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

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.