

EMERGING INFECTIOUS DISEASES®



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January 2015



EMERGING INFECTIOUS DISEASES

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On the Cover

Rogan Brown (1972–) Outbreak, 2014.

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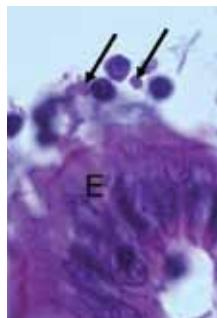
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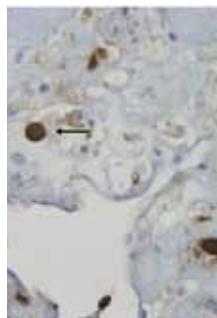
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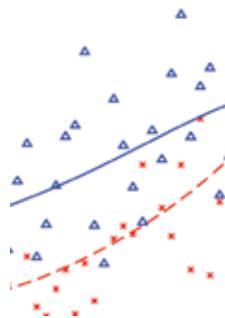
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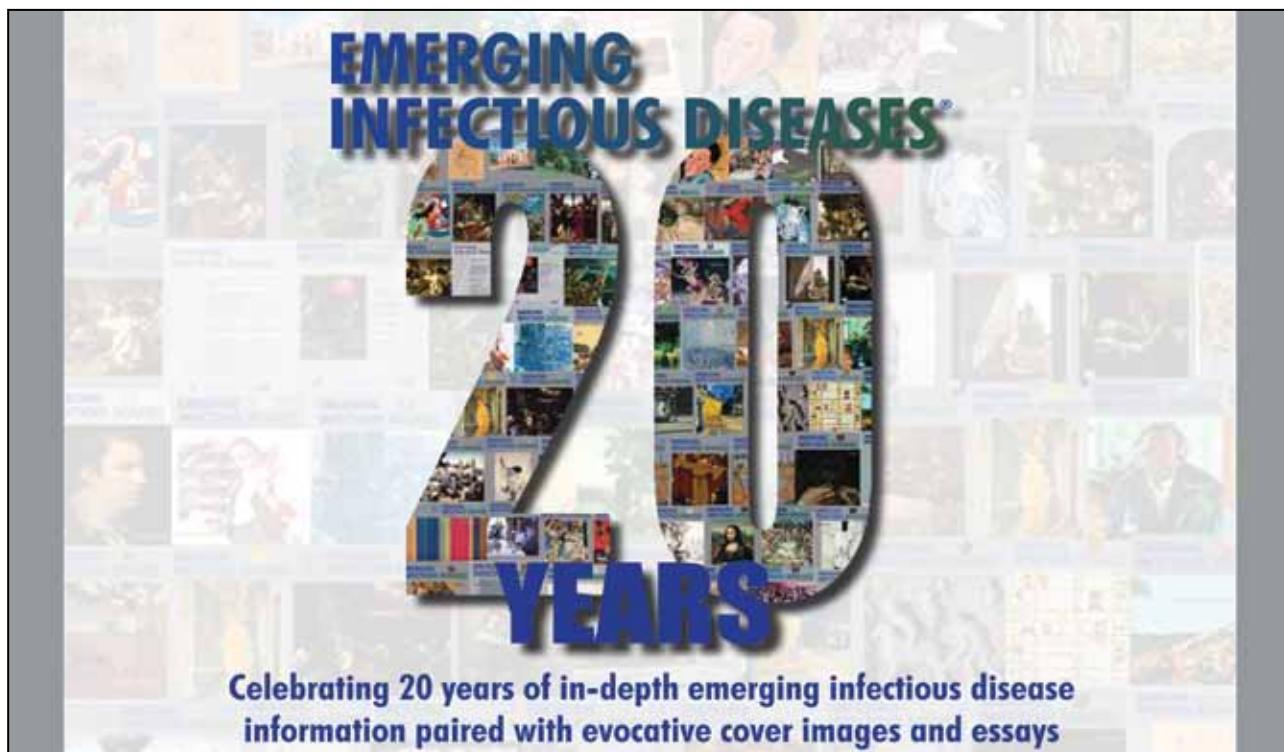
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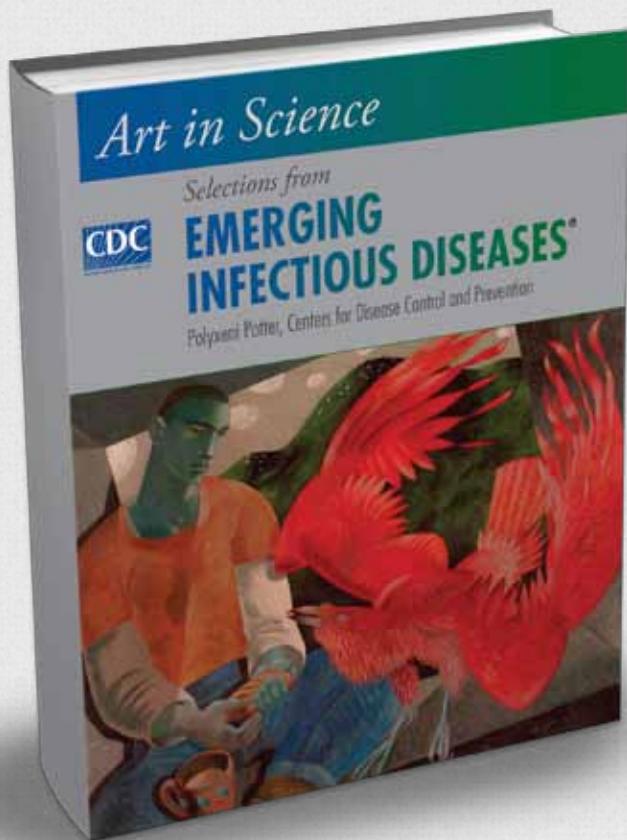
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This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.



Tularemia in Children, Turkey, September 2009–November 2012

Hasan Tezer, Aslınur Ozkaya-Parlakay, Hakan Aykan, Mustafa Erkocoglu, Belgin Gülhan, Ahmet Demir, Saliha Kanik-Yukse, Anil Tapisiz, Meltem Polat, Soner Kara, Ilker Devrim, and Selcuk Kilic

Tularemia, a zoonotic disease caused by *Francisella tularensis*, is found throughout most of the Northern Hemisphere. It is not well known and is often misdiagnosed in children. Our aim with this study was to evaluate the diagnosis, treatment, and prognosis for 100 children with tularemia in Turkey. The mean patient age was 10.1 ± 3.5 years (range 3–18 years), and most (63%) patients were male. The most common physical signs and laboratory findings were cervical lymphadenopathy (92%) and elevated erythrocyte sedimentation rate (89%). Treatment response was higher and rate of relapse lower for children 5–10 years of age than for those in other age groups. Associated with treatment failure were female sex, treatment delay of ≥ 16 days, and use of doxycycline. Tularemia is endemic to Turkey, and the number of cases has been increasing among children as well as adults.

Tularemia, caused by *Francisella tularensis*, is a potentially fatal, multisystemic disease in humans. Tularemia occurs throughout most of the Northern Hemisphere, and the number of cases is increasing in various parts of Europe, especially in the Balkans, Turkey, and Scandinavian countries. There are 4 recognized subspecies of *F. tularensis*, which differ in their pathogenicity and geographic distribution: *tularensis* (type A), *holarctica* (type B), *novicida*, and *mediasiatica*. Among them, subspecies *tularensis* and *holarctica* are of particular clinical and epidemiologic relevance (1–4). Although the highly virulent subspecies *tularensis* is restricted almost exclusively to North America, subspecies *holarctica* is found in Europe, Asia, and North America and represents the most common subspecies involved in human and animal infection (4).

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The clinical forms of tularemia are ulceroglandular or glandular, oculoglandular, oropharyngeal, respiratory, and typhoidal (1). Each form somehow reflects the mode of transmission. The clinical picture and severity of the disease in humans vary considerably depending on the route of infection, the virulence of the causative organism, and the immune status of the host. The ulceroglandular form has been reported as the most prevalent clinical form of the disease in northern Europe, whereas the oropharyngeal form has been most commonly reported in Turkey, Bulgaria, and Kosovo and is attributed to the consumption of contaminated water and food (5–10).

Tularemia is endemic to Turkey, and most cases are reported to occur in late summer or early autumn (10). Various studies on clinical course, treatment, and treatment failure in elderly patients are available in the literature (7,10–12). However, the clinical course of tularemia in children is not well known, and cases in children are often misdiagnosed. Our aim was to demonstrate the clinical features and outcomes for children with tularemia.

Material and Methods

We conducted a retrospective records review for 100 children with a presumptive diagnosis of tularemia who were admitted to the Ankara Hematology Oncology Children's Training and Research Hospital and Gazi University Hospital from September 2009 through November 2012. The diagnosis of tularemia was confirmed by detection of specific antibodies by microagglutination test and/or *F. tularensis* nucleic acid in a clinical specimen (lymph node aspirate).

Microagglutination testing was performed as described (1) by using safranin-stained *F. tularensis* cells from the National Collection of Type Cultures (*F. tularensis* NCTC 10857, *F. tularensis* subsp. *holarctica* live vaccine strain) cells. In brief, 2-fold serial dilutions of serum were incubated overnight with safranin-stained, formalin-killed *F. tularensis* cells at 35°C, and a titer was assigned according to the last well demonstrating full agglutination. According to the legal health regulations in Turkey, a diagnosis of tularemia is serologically confirmed by the presence of at least 1 of the following laboratory findings: 1) compatible clinical signs or symptoms

and specific antibodies at significantly high titers ($\geq 1:160$) and 2) a ≥ 4 -fold increase in 2 successive titers ($\geq 1:160$ for the convalescent-phase titer). Therefore, antibody titers $\geq 1:160$ were considered to be positive.

DNA from clinical samples was extracted by using a commercial purification system with columns (QIAamp DNA extraction mini kit; QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Clinical samples from patients with suspected tularemia were screened for evidence of the tularemia agent by using conventional PCR.

Affiliation with the genus *Francisella* was confirmed by amplification of the 17-kDa outer membrane lipoprotein gene fragment as described by Sjöstedt et al. (13). Primers TUL4-435 (5'-GCT GTA TCA TCA TTT AAT AAA CTG CTG-3') and TUL4-863 (5'-TTG GGA AGC TTG TAT CAT GGC ACT-3'), which amplify a 420-bp fragment of the 17-kDa lipoprotein, were used. Samples were added to a PCR mixture containing a final concentration of 200 mmol/L (each) deoxynucleoside triphosphate mixture, 10X Hot Start PCR Buffer (Fermentas/Thermo Scientific, Vilnius, Lithuania), 0.4 mmol/L (each) primer (Ella Biotech GmbH, Martinsried, Germany), 2.5 mmol/L $MgCl_2$ (Fermentas/Thermo Fisher Scientific), 1 mL bovine serum albumin (1 mg/mL, Sigma-Aldrich), and 1.25 U *Taq* DNA polymerase (Fermentas/Thermo Fisher Scientific) in a total reaction volume of 50 mL. The reaction was performed in a DNA thermal cycler (Thermo Hybaid OMN-E, Ashford, UK) at a denaturation temperature of 94°C for 4 min and was followed by 40 cycles at 94°C for 40 s, 64°C for 30 s, and 72°C for 45 s, and final extension at 72°C for 5 min. The PCR products were sized on agarose gels and stained with ethidium bromide.

After confirmation of isolates as *F. tularensis* by PCR with *tul4* primers, conventional PCR selective for the region of difference - 1 was used to determine subspecies identity (14). PCR for detection of *Mycobacterium tuberculosis* complex was also performed on lymph node aspirates. Patients with abdominal pain were evaluated by abdominal ultrasonography.

Treatment options varied according to the patient's age. One of the following regimens was administered: gentamicin (5 mg/kg 2 or 3 times daily, intravenously or intramuscularly) for 10 days, doxycycline (100 mg orally 2×/day) for 14 days, ciprofloxacin (20 mg/kg 2×/day) for 10–14-days, or streptomycin (30–40 mg/kg/day, divided into 2 doses/day, intramuscularly) for 10 days. Doxycycline was not the drug of choice for patients younger than 8 years.

Good response was defined as complete recovery from the illness after treatment with antimicrobial drugs only (no suppuration, no need for surgical procedures, and no relapse). Therapeutic failure was defined as the presence of 1 of the following: increased lymph node size, occurrence of new lymphadenopathy, or both; persistent or recurrent

fever; and constantly elevated or increasing acute-phase reactants (erythrocyte sedimentation rate and C-reactive protein levels).

Statistical analyses were performed by using SPSS version 20 (SPSS Inc., Chicago, IL, USA). Numerical variables are expressed as means (or medians) and standard deviations. Categorical variables are expressed as frequencies and percentages. For categorical variables, if the χ^2 condition was met, multiple group comparisons were performed by χ^2 test; if not, Monte Carlo simulation was used in multiple group comparisons and the Fisher exact test was used to compare 2 groups. Numerical variables with normal distribution were compared by using the Student *t*-test, and those with nonnormal distribution were compared by using the Mann–Whitney U test. Logistic regression analysis (method = ENTER) was used to determine the risk factors. A p value <0.05 was considered to be significant.

Results

A total of 100 children with laboratory-confirmed tularemia were included in the study. Most (63%) of the children were male. Mean patient age was 10.1 ± 3.5 years (range 3–18 years); half (50%) were younger than 10 years, and only 9% were younger than 5 years (Figure 1). Most (96%) patients were from Ankara Province, and only 4% were from the Aegean region. Most (62%) patients lived in villages in Ankara, and the rest (16%) came from an area ≈ 250 km in diameter. More than half (60%) of the patients were admitted to the hospital during winter and autumn (Figure 2). In terms of risk factors, in addition to tap water, natural stream water was available and had been widely used for drinking and cooking; 76% of the patients had a history of drinking water from these sources. Contact with rodents was reported for 32% of patients, and 44% of patients had at least 1 neighbor who had been exposed to rodents. None of the patients had a history of tularemia.

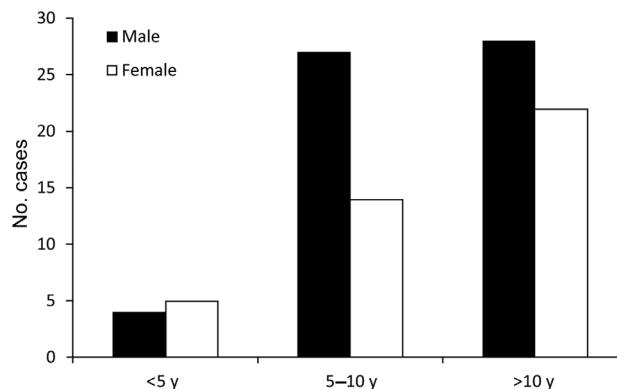


Figure 1. Diagnosis of tularemia for 100 children, by patient age group and sex, Ankara, Turkey, September 2009–November 2012.

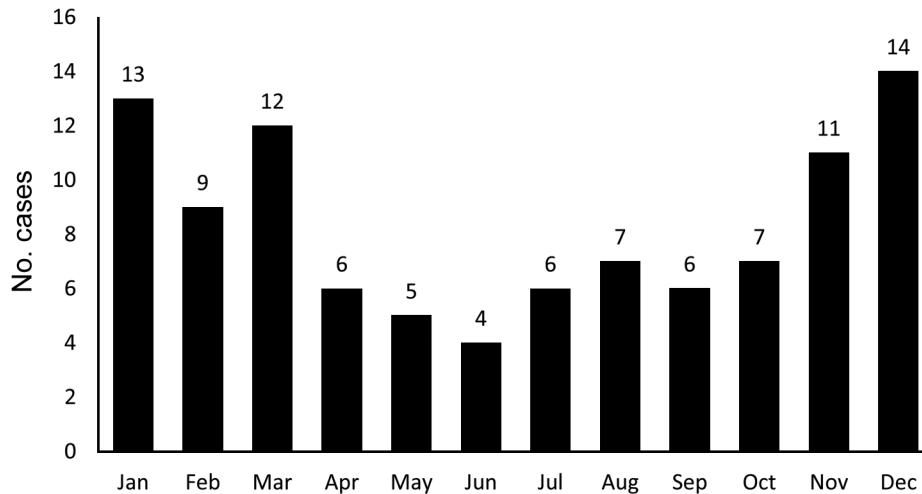


Figure 2. Diagnosis of tularemia for 100 children, by month, Ankara, Turkey, September 2009–November 2012.

Clinical Findings

The most common clinical signs were swelling on the neck (cervical lymphadenopathy) (92%), fever (63%), and tonsillitis (55%) (Table 1). Abdominal lymphadenopathy was found in 3 patients. Oropharyngeal tularemia was diagnosed for 90 patients, ulceroglandular tularemia for 7, and oculoglandular tularemia for 3 (Table 1). No patient died during or after therapy.

During the initial visits, skin rashes were found on 6 patients; 4 of these patients had papular lesions on the extremities, and the other 2 had erythema nodosum without associated symptoms of tularemia. During follow-up visits, 1 of the patients with erythema nodosum had oculoglandular tularemia and the other had oropharyngeal tularemia.

Laboratory Findings

Diagnoses were primarily established by microagglutination test results (titer $\geq 1:160$) for 98 patients and by *F. tularensis*-specific PCR analysis for 2 patients. The microagglutination method revealed titers of 1:40–1:2,560. The median agglutinin titer was 1:810. Paired serum samples from 2 patients with low acute-phase titers revealed a 4-fold increase in titer.

The other abnormal laboratory findings were elevated leukocyte counts and erythrocyte sedimentation rates. Mean leukocyte count was $11,918 \pm 3,924/\text{mm}^3$ (range 5,600–23,800/ mm^3). Erythrocyte sedimentation rate was elevated for 89% of the patients (mean 60.13 \pm 27.5 mm/h, range 8–120 mm/h), and for 68% of patients the rate was >50 mm/h. For 18 patients, only C-reactive protein was elevated (median 2.51 mg/dL, range 0.1–19.2 mg/dL). Ultrasonography was performed for 89 patients with lymphadenopathy and for 77 of these patients revealed cystic necrotic abscesses characterized by central hypoechoogenicity and septations. No significant difference was found between erythrocyte sedimentation rate

(>50 mm/h vs. <50 mm/h) and C-reactive protein levels, respectively, of patients with good response ($p = 0.133$, $p = 0.819$), those who needed surgical procedures ($p = 0.131$, $p = 0.103$), those who experienced spontaneous suppuration ($p = 0.448$, $p = 0.674$), and those who experienced relapse ($p = 0.325$, $p = 0.963$).

Treatment Response

No deaths occurred during the 12-month follow-up period. Of 62 patients in the treatment failure group, previous medications included β -lactams/macrolides. Another 2 patients had received a diagnosis of tuberculosis according to pathologic findings and were receiving antituberculosis treatment at the time of diagnosis.

Initial therapy consisted of intravenous gentamicin (56 patients), oral doxycycline (23 patients), oral ciprofloxacin (20 patients), or intramuscular streptomycin (1 patient). The duration of antimicrobial drug treatment was 10–14 days. Relapse rates were similar for patients who received gentamicin, doxycycline, or ciprofloxacin ($p = 0.306$).

Table 1. Clinical characteristics of 100 children with tularemia, Ankara, Turkey, September 2009–November 2012*

Characteristic	No. children
Clinical sign	
Cervical lymphadenopathy	92
Unilateral	73
Bilateral	19
Abdominal lymphadenopathy	3
Tonsillitis	55
Fever	63
Conjunctivitis	5
Ulceration	5
Diarrhea	4
Erythema nodosum	4
Disease form	
Oropharyngeal	90
Ulceroglandular	7
Oculoglandular	3

*Mean patient age was 10.1 ± 3.5 years; 63 boys, 37 girls.

Complete recovery with no complications (e.g., suppuration, need for surgical procedures, or relapse) occurred for 54 patients. However, for 8 patients with a good response, although surgery was not needed, a surgical procedure was performed to hasten the healing process. While receiving specific antimicrobial drug therapy, 9 patients experienced spontaneous suppuration. During the 12-month follow-up period after initial treatment (antimicrobial drug alone or with surgical procedures), 74 patients neither required re-treatment nor experienced relapses. A surgical procedure was performed for 43 (58.1%) of these 74 patients. The remaining 26 patients experienced relapses that required a second course of therapy (Table 2). All 26 patients received antimicrobial drugs, and 17 underwent surgical procedures. Among these 17 patients, 10 underwent a second surgical procedure. All 17 patients recovered completely and did not experience any relapses during the 12-month follow-up period. Outcome data are shown in Figure 3. Excisional biopsy specimens from 15 patients underwent histopathologic examination and revealed chronic necrotizing lymphadenitis with histiocytic infiltration and caseous necrosis.

Factors Predicting Response to Treatment

Age

Complete recovery rate (good response) was higher among children 5–10 years of age than among those in the other age groups (<5 and >10 years) (p = 0.018) (Table 2). The need for surgical procedures (p = 0.36) and spontaneous suppuration of lymph nodes (p = 0.46) were similar for children in all age groups. Rates of relapse were significantly lower for children 5–10 years of age than for those in the other age groups (p = 0.03) (Table 2).

Treatment Delay

For 56 patients, the time from diagnosis to treatment initiation was >2 weeks. Good response rate (p = 0.02) was significantly lower and relapse rate was significantly higher (p = 0.03) among patients for whom treatment was delayed ≥16 days, and spontaneous suppuration rate (p = 0.23) and need for surgical procedures (p = 0.12) were similar among

patients for whom treatment was delayed ≥16 days and those for whom treatment was delayed <16 days (Table 2).

According to regression analysis, significant factors for treatment failure were female sex, treatment delay ≥16 days, and doxycycline use. The significant factor for spontaneous suppuration was treatment delay ≥16 days (Table 3).

Discussion

Tularemia is endemic to Turkey. Although most studies have examined adult populations, tularemia can occur in patients of all ages and is more prevalent among children 5–9 years of age and in persons older than 75 years (6,10–12,15–17). Ulceroglandular tularemia is the most commonly reported clinical form throughout the world (1,4,5,18). However, prevalence of oropharyngeal tularemia is higher in Turkey, Bulgaria, Kosovo, and Norway (6,7,10,15–17,19), as is reported here.

In studies reported from Turkey, cases of tularemia in patients younger than 10 years were extremely rare; in a report of 61 tularemia cases, no patient was younger than 10 years, and in another study, only 2 patients were younger than 10 years (11,17). In our study, half of the patients were younger than 10 years. The prevalence of tularemia among children probably tended to increase because of increased exposure of farmers’ infants and school-aged children to farm animals, rodents, or rodent excreta while helping their families with farming and drinking nonchlorinated (natural spring) water.

The source of infection and the mode of transmission often remain elusive for patients with tularemia. However, the high prevalence of oropharyngeal tularemia has been associated with the consumption of contaminated water (6,10–12). Tularemia outbreaks that are associated with the consumption of hunted animals usually occur in summer and early autumn (1,3–5), whereas waterborne tularemia outbreaks usually occur in autumn and winter (10–12,20–22). Similar to cases in adults, almost all of the cases in children in our study occurred in winter or autumn.

F. tularensis subsp. *holarctica* usually causes mild infections with low mortality rates. For patients with tularemia, physical examination usually reveals unilateral cervical lymphadenopathy, tonsillitis, and fever. The most commonly

Table 2. Outcomes for 100 children with tularemia, Ankara, Turkey, September 2009–November 2012

Variable	Treatment response, no. (%) children			
	Good response, n = 54	Spontaneous suppuration, n = 9	Relapse, n = 26	Needed surgical procedure, n = 43
Age group, y				
<5, n = 9	4 (44.4)	1 (11.1)	4 (44.4)	3 (33.3)
5–10, n = 41	29 (70.7)	2 (4.9)	5 (12.2)	15 (36.6)
>10, n = 50	21 (42)	6 (12)	17 (34)	25 (50)
p value	p = 0.02	p = 0.46	p = 0.03	p = 0.36
Treatment delay, d				
<16, n = 60	38 (63.3)	13 (21.7)	2 (3.3)	22 (36.7)
≥16, n = 40	16 (40.0)	13 (32.5)	7 (17.5)	21 (52.5)
p value	p = 0.02	p = 0.23	p = 0.03	p = 0.12

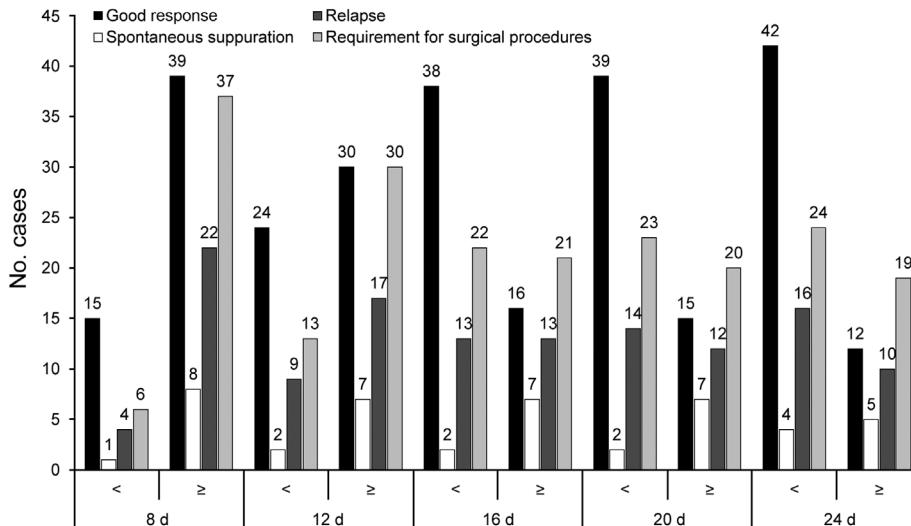


Figure 3. Data regarding outcome for 100 children with tularemia, Ankara, Turkey, September 2009–November 2012.

reported finding in tularemia patients is lymphadenopathy (6,11,12,22). In our study, the most common (92%) complaint at admission was a mass in the neck region (cervical lymphadenopathy), followed by tonsillitis and fever.

Secondary skin manifestations of tularemia are erythema multiforme, erythema nodosum, and papular lesions. Skin manifestations might occur in all forms of tularemia and probably result from the systemic spread of organisms (23,24). In our study, erythema nodosum was observed in only 2 (2.6%) patients, less than that observed in other studies (6–9,11,15). Of note, none of the patients had any signs or symptoms suggesting tularemia at illness onset. In a study of 50 patients in Finland, the frequency of skin lesions was ≈50% (25). In our study, the frequency of skin lesions was as low as 6%.

Routine peripheral blood counts do not provide a diagnostic clue for tularemia. The standard test for diagnosis of tularemia is culture, which is difficult and requires a Biosafety Level 3 facility (1). Therefore, other methods, mainly serologic testing and PCR, have generally been preferred for the diagnosis of this disease (26–29). The most frequently ordered diagnostic testing for *F. tularensis* infection is serology. In Turkey, antibody titers of ≥1:160 obtained by microagglutination testing are generally consistent with infection (6,10–12,21,30). However, antibody levels do not usually increase until the second week of illness (10,30). In addition, the existence of antibody-negative but culture-positive patients has been reported in the literature (30). In another study, for some tularemia patients with negative microagglutination test results, diagnosis was made only

Table 3. Results of regression analysis for variables predicting treatment outcomes for 100 children with tularemia, Ankara, Turkey, September 2009–November 2012*

Variable	Treatment failure		Spontaneous suppuration		Relapse		Requirement for surgical procedures	
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
Female sex	3.449 (1.250–9.514)	0.017	1.348 (0.313–5.804)	0.689	2.314 (0.850–6.300)	0.101	1.842 (0.716–4.742)	0.205
Treatment delay ≥16 d	3.277 (1.220–8.799)	0.019	6.074 (1.185–31.131)	0.030	1.295 (0.477–3.517)	0.612	1.876 (0.741–4.751)	0.184
Antimicrobial drug								
GEN†		0.067	ND	ND		0.643		0.140
DOX	4.965 (1.280–19.264)	0.021	ND	ND	1.295 (0.355–4.733)	0.695	3.308 (0.957–11.428)	0.059
CIP	2.637 (0.733–9.483)	0.138	ND	ND	0.542 (0.124–2.371)	0.416	2.689 (0.808–8.950)	0.107
GEN + DOX	0.458 (0.060–3.473)	0.450	ND	ND	0.376 (0.035–4.033)	0.419	0.636 (0.094–4.317)	0.643
Age at diagnosis	0.926 (0.832–1.031)	0.162	0.941 (0.755–1.173)	0.589	0.925 (0.801–1.067)	0.283	0.935 (0.841–1.041)	0.219
Age at study inclusion	2.611 (0.739–9.222)	0.136	2.096 (0.151–29.018)	0.581	2.588 (0.476–14.086)	0.271	2.321 (0.658–8.183)	0.190

*Because the number of patients was limited, antimicrobial drugs were not included in the regression analysis for spontaneous suppuration. CIP, ciprofloxacin; DOX, doxycycline; GEN, gentamicin; ND, not done.
†Reference variable.

by ELISA or PCR (28). In our study, microagglutination titers were $\geq 1:160$ for 98 patients, and PCRs were positive for 2 patients with negative serologic test results. In the literature, the sensitivity and specificity of microagglutination testing, based on the results obtained for samples from healthy persons and patients with illnesses other than tularemia, were 97.6% and 98.7%, respectively (30).

In our study, 92 patients had cervical lymphadenopathy. Despite negative microagglutination test results, PCRs for *F. tularensis* in the tissue were positive. The patients recovered fully after taking antimicrobial drugs specific for tularemia. Thus, in our opinion, although microagglutination testing had been recommended for the diagnosis of tularemia, PCR testing should be considered for patients with signs and symptoms strongly suggestive of tularemia and with negative microagglutination test results.

PCR testing has been performed on samples (lymph node aspirates) collected during epidemics in Turkey (2009–2012) (12,31,32). The sensitivity of PCR ranged from 33.9% to 91.0% for patients with culture- and/or serology-verified oropharyngeal and glandular tularemia, depending on the duration of illness, and PCR was more sensitive than culture of lymph node aspirates (63.3% vs. 23.3%) (12,31,32). PCR is the most rapid and sensitive test available for detecting *F. tularensis* in patient specimens (26).

Beta-lactams, cephalosporins, and clindamycin are considered to be ineffective for the treatment of *F. tularensis* infections (5,6). In the United States and Europe, treatment with aminoglycosides has been the mainstay of treatment for children with tularemia (3,33,34). Because of the possible ototoxic side effects of aminoglycosides, evaluation of hearing function before treatment and monitoring of inner ear function during treatment are highly recommended (1,11). Expert groups in Europe recommend quinolones as an effective treatment alternative for subsp. *holarctica* infections (29,33). In our study, ciprofloxacin was preferred for 26 patients. No relapses occurred in these patients, although our sample size was not large enough to enable us to draw definitive conclusions. Further randomized studies in large patient populations are needed. Tetracyclines should be administered for at least 14–21 days because relapses in patients receiving these bacteriostatic agents have been reported (11). In patients younger than 8 years, the lack of alternative oral medications for tularemia constitutes a major problem. A study that evaluated 67 cases in children established that the time from symptom onset to diagnosis was 26 days (range 8–92 days). Delayed treatment was explained by the use of antimicrobial drugs not effective against *F. tularensis* in at least 20 patients. For some patients, although tularemia was suspected, treatment delays were attributed to lack of oral alternatives to aminoglycosides (34). Duration of treatment depends on the clinical course of the

disease. In our study, the relapse rates were similar among patients receiving each of the 4 antimicrobial drugs, including doxycycline.

Delayed initiation of antimicrobial therapy was reported to be strongly associated with treatment response. In a recent study from Turkey that included adults, initiation of therapy after 14 days was reported to be associated with treatment failure (29) as it was in previous reports (9). In our study, factors that significantly affected treatment failure were female sex, treatment delay ≥ 16 days, and doxycycline use.

Recently a small-scale study from Turkey, which included children, reported that all children responded well to treatment regardless of age (17). However, in our study, response to treatment was better and rates of relapse were lower among children 5–10 years of age than among children in other age groups. This finding may be the target of immunopathogenesis research.

In conclusion, our study indicated that tularemia affects children differently than adults, suggesting that tularemia should be kept in mind as a diagnosis for children with severe lymphadenopathy and tonsillitis who show no response to β -lactam antimicrobial drugs. Additionally, according to our results, prognosis varied by age, and treatment failure was associated with female sex, treatment delay ≥ 16 days, and doxycycline use.

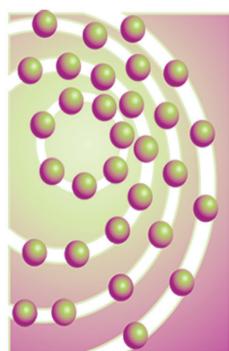
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Pneumonic Plague Outbreak, Northern Madagascar, 2011

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Yersinia pestis, the causative agent of plague, is endemic to Madagascar, particularly to the central highlands. Although plague has not been previously reported in northern Madagascar, an outbreak of pneumonic plague occurred in this remote area in 2011. Over a 27-day period, 17 suspected, 2 presumptive, and 3 confirmed human cases were identified, and all 15 of untreated patients died. Molecular typing of *Y. pestis* isolated from 2 survivors and 5 *Rattus rattus* rat samples identified the Madagascar-specific 1.ORI3-k single-nucleotide polymorphism genotype and 4 clustered regularly interspaced short palindromic repeat patterns. This outbreak had a case-fatality rate of 100% for nontreated patients. The *Y. pestis* 1.ORI3-k single-nucleotide polymorphism genotype might cause larger epidemics. Multidrug-resistant strains and persistence of the pathogen in natural foci near human settlements pose severe risks to populations in plague-endemic regions and require outbreak response strategies.

Yersinia pestis is the causative agent of plague, a severe and life-threatening zoonotic disease. During 3 pandemics, different genotypes of this bacterium spread to various countries and caused millions of deaths (1–6). *Y. pestis* genotype 1.ORI3 of the biovar Orientalis was introduced to Madagascar in 1898 during the third pandemic and has persisted there endemically ever since (6,7). Worldwide, an average of 4,000 human plague cases is reported each year. Madagascar is one of the most active plague foci and has an annual average of 1,500 confirmed cases (8,9). The pathogen emerges in multiannual cycles; a peak of pathogen prevalence was observed in Madagascar in 1997 and caused ≈3000 cases, 20 times more than in 1994 (126 cases) (9).

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Bubonic plague, the most common form of plague, results from the bite of an infected flea. The infection may spread hematogenously and cause secondary pneumonic plague. If the pathogen is transmitted as an aerosol by droplets or by contaminated dust, primary pneumonic plague may result. After a latency period of 1–5 days, pneumonic plague progresses to the stage of hemoptysis. At this lethal stage of the disease, which lasts ≤3 days, patients are highly infectious (10,11). Plague can be treated with antimicrobial drugs if diagnosed early and if caused by a drug-sensitive strain (9).

Worldwide, only a few pneumonic plague outbreaks have been reported (12–15). However, to understand the epidemic potential of *Y. pestis*, extensive outbreak analyses are essential. In 1997, a pneumonic plague outbreak occurred in Madagascar near the capital of Antananarivo (13). Health authorities responded immediately, and strain cultivation was successful. Three other pneumonic plague outbreaks have been reported, 1 in Uganda (2004) and 2 in the Democratic Republic of Congo with 87 cases (2005) and 117 cases (2006), respectively (14,15). During the 1920s–1930s, valuable descriptions of 2 plague epidemics in Manchuria, China (≈10,000 cases) were reported (10,12).

In this report, we describe an outbreak and highly progressive spread of pneumonic plague in northern Madagascar, a remote region that was supposedly free of *Y. pestis*, in 2011. We investigated whether *Y. pestis* might cause larger outbreaks or epidemics with high case-fatality rate within a short period.

Outbreak Progression and Investigation

The outbreak investigation protocol was approved by the Ethical Committee, Ministry of Health of Madagascar. The outbreak area contained 7 villages along a field path in the communes of Ambarakaraka and Anaborano, Ambilobe District, in northern Madagascar at an altitude <500 m. In January 2011, two brothers (boys) were working in a copper mine in Beramanja, Madagascar. On January 6, the boys returned home to Ankatakata (distance 50 km) (Figure 1).

During the journey, fever, headache, and chills developed in 1 of the boys (age 13 years). Subsequently, he experienced severe chest pain, a deep cough, and hemoptysis, which are the clinical signs of pneumonic plague. He died at home on January 14 (Figure 2). It can be assumed retrospectively that this boy (index case-patient 1) had been infected with *Y. pestis* in Beramanja or during the journey home (Figures 1, 2). On January 21, his mother, who had provided him with treatment until he died, also died (case-patient 2 in household A). Her husband (case-patient 3), daughter (case-patient 4), and granddaughter (case-patient 5) died on January 22. Symptoms of pneumonic plague had developed in these 3 persons (Figure 2; Table 1). These 3 persons were provided treatment by neighbors in household B (Figure 2).

On January 26, a second wave of human pneumonic plague cases was observed in 12 other persons (Figure 2; Table 1). During this second wave, household A (case-patients 6, 7, 12, 13, and 16) and household B (case-patients 8, 10, 11, 14, and 17) in Ankatakata were affected. Case-patient 9 was probably infected while visiting her sister-in-law (case-patient 2) in Ankatakata and transmitted the pathogen to her home (household C) in Antsakoabe (Figures 1, 2). Case-patients 15 and 18 were also present in Ankatakata and transmitted the infection to Tsaratanana (household D) and Ankiganyo (household E). Case-patient 18 was responsible for a third small wave of pneumonic plague that started on January 31 and spread to Ambarakaraka (Figure 2; Table 2). This case-patient infected her brother (case-patient 19), who had carried his sister to a traditional healer (case-patient 20). Symptoms of pneumonic plague developed in all 3 persons; all 3 died (Figure 2; Table 1).

Contacts

During our epidemiologic investigation, 41 contact persons were identified. These persons had interacted with the patients but did not show specific symptoms of pneumonic plague. All provided a serum sample for diagnostic testing (Table 2). Sixteen family contacts (c1–c16) were defined as persons who lived in the same household as an infected person during the outbreak (Figure 2; Table 2). Twenty-five other contacts (c17–c41) had spent some time with a patient or approached a patient who died during the outbreak (Figure 2; Table 2).

In Ankatakata, we found 5 family contacts (c1–c5) in households A and B who had received chemoprophylaxis on January 28. In Antsakoabe, case-patient 9 died in her home on January 26. Her husband and 3 children (c6–c9) lived in the same house. They received chemoprophylaxis on February 6 and provided serum samples on February 15. In Tsaratanana, the wife (c10) of case-patient 15 refused chemoprophylaxis. In Ankiganyo, 4 family contacts (c11–c14) of deceased case-patients 18 and 19 received chemoprophylaxis on February 6. Contact c14 was

seropositive for pneumonic plague. In Ambarakaraka, 2 family contacts (c15 and c16) of case-patient 20 received chemoprophylaxis on February 9 (Figure 2; Table 2).

The status of nonfamily contacts was as follows. Case-patient 10 moved to Mandriavary, which is 8 km from Ankatakata, and died there of suspected pneumonic plague. We identified 9 contact persons (c17–c25) in Mandriavary. All except 1 (c25) had received chemoprophylaxis on January 28 and showed seronegative results. Contact 25 was a 21 year-old man who had carried case-patient 3 in Ankatakata. He had a cough and was seropositive (Table 2; Figure 2). Case-patient 11 died in Antafiabe. He had contact with 6 other persons (c26–c31). These 6 persons received chemoprophylaxis on January 28. Although 1 contact (c26) shared her bed with case-patient 11 until his death on January 27, these 2 persons did not show clinical signs or symptoms or seroconversion. We found 10 additional contacts (c32–c41), who had attended funerals for case-patients in

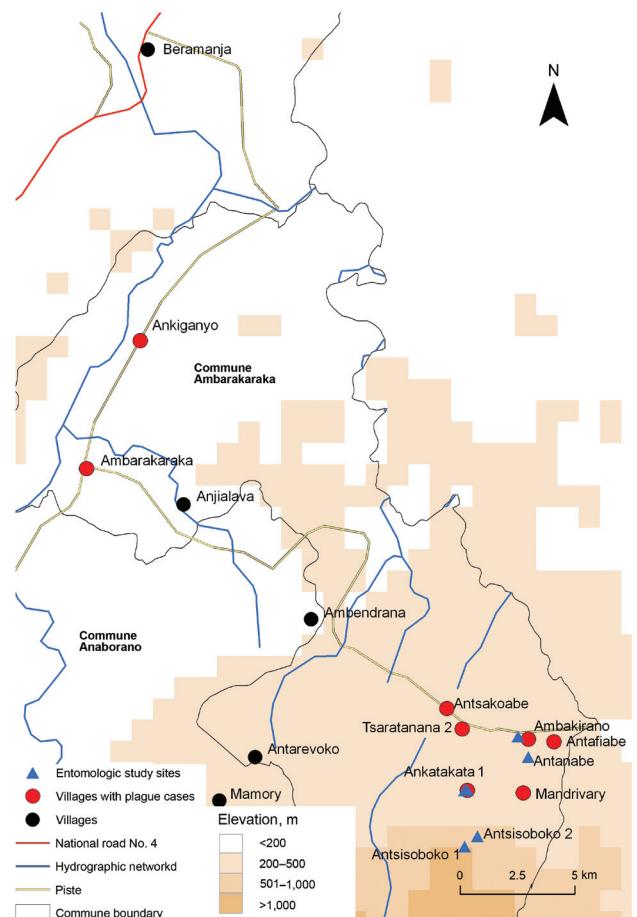


Figure 1. Location of pneumonic plague outbreak in the communes of Ambarakaraka and Anaborano, northern Madagascar, 2011. A copper mine is located in Beramanja. The index case-patient was infected with *Yersinia pestis* on the 80-km trail (piste) to Ankatakata.

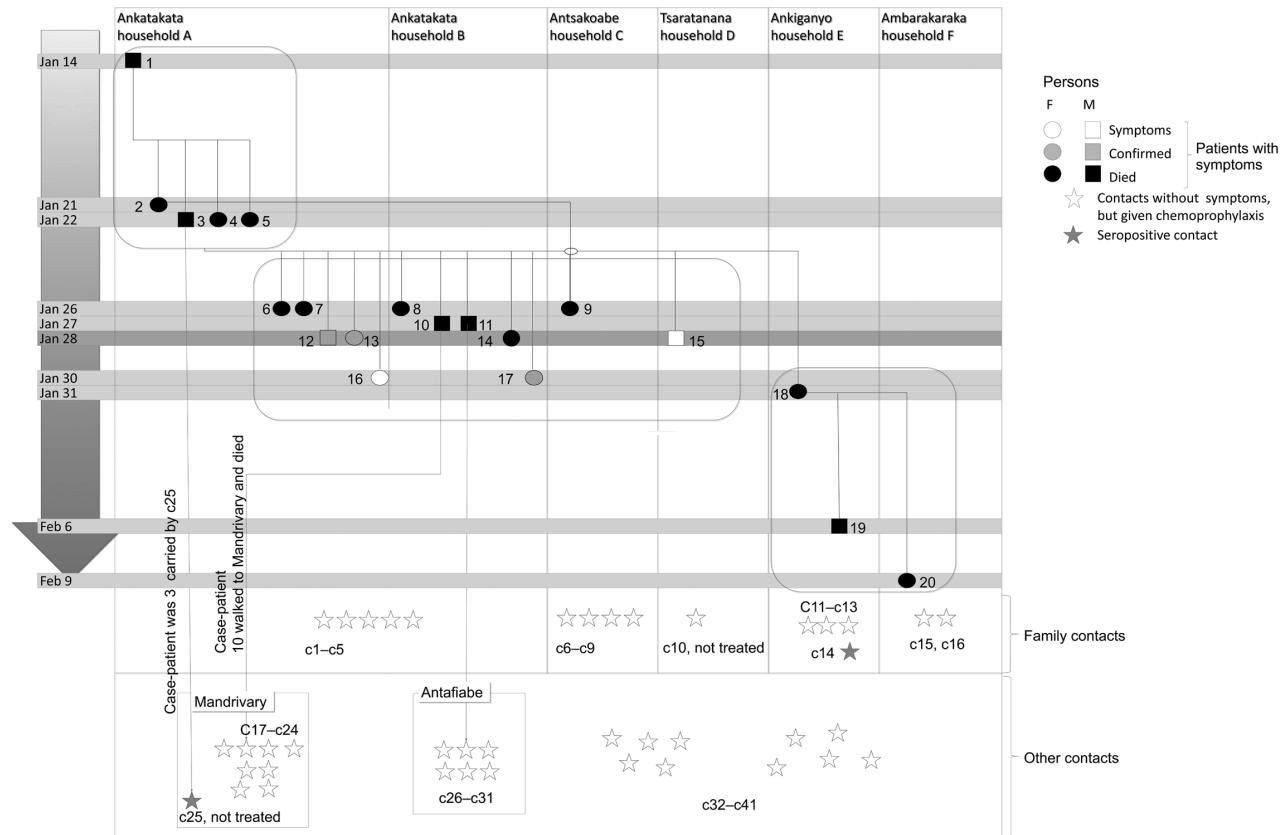


Figure 2. Infection pattern during pneumonic plague outbreak, northern Madagascar, 2011. The outbreak spread to other neighboring villages during January 14–February 9. Twenty persons in 6 households (A–F) in 5 villages had symptoms of pneumonic plague. The outbreak population was divided into 3 groups (group 1: case-patients 1–5; group 2: case-patients 6–17; and group 3: case-patients 18–20). Patients received treatment by January 28. Because of geographic distance, none of the patients in group 3 received treatment. Contacts were divided into family contacts (c1–c16) who lived in an affected household and other contacts (c17–c41) who interacted with infected patients or patients who died. All contacts, except c10 and c25, received antimicrobial drug prophylaxis. Two contacts (c14 and c25) were seropositive (single serum sample); all other contacts remained seronegative.

different villages. Three of these contacts had mild pulmonary infection, but had not consulted a physician and were seronegative (Figure 2; Table 2).

Diagnosis and Case Assessment

Two weeks after the death of the index case-patient on January 28, health personnel arrived in Ankatakata. Treatment with antimicrobial drugs was provided to 5 patients (case-patients 12, 13, 15, 16, and 17), and chemoprophylaxis was provided to contact persons and health personnel according to recommendations of the World Health Organization (WHO) (9) (Figure 2; Tables 1, 2). Because we did not know that the outbreak was ongoing in Ankiganyo (30 km from Ankatakata), treatment was not provided to case-patients 18, 19, and 20 (Figures 1, 2). It was only after these 3 case-patients died that contacts of these patients received chemoprophylaxis (Figure 2; Table 2).

Because persons who died were not sampled post-mortem, only a limited number of clinical samples were

collected (Table 1). Of collected sputum samples, 2 (from case-patients 12 and 13) were positive for *Y. pestis*-specific F1 antigen by immunochromatographic rapid dipstick test (Table 1) (16). All samples were subsequently transported (>900 km) to the WHO Collaborating Center for Plague at the Institut Pasteur de Madagascar in Antananarivo. Sputum samples were incubated in bacterial culture media and inoculated into laboratory mice. However, isolation of *Y. pestis* was not successful (Tables 1, 3). Collected serum samples were analyzed by using a *Y. pestis*-specific F1 antigen IgG ELISA as described (17). Positive samples were quantified by using serial dilutions. Three of 5 paired serum samples (from case-patients 12, 13, and 17) had 4-fold increases in titer in the second serum (Table 1). Of samples from contact persons, 2 of 41 single serum samples showed a positive result (Table 2).

Subsequent case assignment was conducted according to WHO recommendations for plague-endemic countries (18). Three types of cases were identified: suspected cases (specific

clinical symptoms), presumptive cases (positive serologic result for antibody against F1 antigen), confirmed cases (4-fold increase in titer of antibody against F1 antigen in paired serum samples or a positive culture result) (18). When we applied these recommendations to the outbreak, we identified 17 suspected cases (in case-patients 1–11, 14–16, and 18–20), 2 presumptive cases (in c14 and c25), and 3 confirmed cases (in case-patients 12, 13, and 17) (Tables 1,2).

All 20 patients with pneumonic plague had sudden onset of fever, cough, hemoptysis, and chest pain. The latency period was 4–6 days, and the infectious period was 48–72 hours (Figure 2). When given antimicrobial drugs, 5 patients (case-patients 12, 13, 15, 16, and 17) survived (Table 1). In contrast, the 15 case-patients who were not treated died of pneumonic plague. Of the 36 persons living in affected households A–F, 20 showed specific symptoms and 15 died. The overall attack rate was 55%, and the case-fatality rate was 75% (Figure 2).

Outbreak Focus

Because of poor accessibility to the remote area of the outbreak and thunderstorms, a field investigation on the plague focus was not started until April 1, which was 2 months after the outbreak (Table 3). No dead rats were observed before, during, and after the outbreak, which is an unusual finding for a plague epidemic in Madagascar.

The sampling sites were chosen in the 2 villages (Ambakirano and Ankatakata) and surrounding woodlands of Antsisoboko and Antanabe (Figure 1). A total of 36 traps were set during 30 nights. Sixty-four rodents were trapped: 51 black rats (*Rattus rattus*), 3 house mice (*Mus musculus*), 6 greater hedgehogs (*Setifer setosus*), 3 short-tailed shrews (*Microgale*

brevicaudata), and 1 Asian house shrew (*Suncus murinus*). Serum or spleen samples were obtained from these 64 rodents and from 5 dogs (Table 3). Pathogens were not isolated from these animals. Fleas were not detected in flea-specific traps.

Bacteriologic culture, serologic analysis, and molecular testing were conducted as described for human samples. Bacteriological culture results was negative, but 1 *S. setosus* hedgehog and 2 dogs were seropositive for IgG against F1 by ELISA (Table 3).

Molecular Investigation

Molecular diagnostics of *Y. pestis* DNA was performed by using a PCR specific for the *Y. pestis* plasminogen activator and capsule antigen fraction 1 genes as reported (19,20). Samples from case-patients 12 and 13 showed positive results for *Y. pestis* DNA (Table 3). For comparison, samples from person with confirmed human plague were also analyzed: 1 each from Mandritsara (2010) and Bealanana (2011) (400 km from Ambilobe) and 1 from Ankazobe (2010) (800 km from Ambilobe) (Table 4). Of animal spleen samples collected, 5 samples from *R. rattus* rats were positive for *Y. pestis* DNA (Table 3).

We analyzed 14 canonical single-nucleotide polymorphisms (SNPs) to determine the SNP genotype (phylogenetic position) of 10 *Y. pestis*-positive samples within the 1.ORI3 group (s232, s1362, s1375, s190, s197, s1367, s1004, s1025, s1377, s1089, s1363, s206, s1373, and s152) according to a hierarchical molecular typing approach (1). All 10 samples showed an identical 1.ORI3-k SNP pattern; we also found 1 derived SNP (s232) and 13 ancestral SNPs (Table 4).

In a second typing approach, *Y. pestis*-specific clustered regularly interspaced short palindromic repeats

Table 1. Diagnostic, epidemiologic, and molecular data for persons with symptoms of pneumonic plague during pneumonic plague outbreak, northern Madagascar, 2011*

Patient/household	Received antimicrobial drug		Sampling	RDT	Serologic analysis	Culture	WHO case definition	Molecular analysis
	treatment	and survived						
1/A	No	No	No	ND	ND	ND	Suspected	ND
2/A	No	No	No	ND	ND	ND	Suspected	ND
3/A	No	No	No	ND	ND	ND	Suspected	ND
4/A	No	No	No	ND	ND	ND	Suspected	ND
5/A	No	No	No	ND	ND	ND	Suspected	ND
6/A	No	No	No	ND	ND	ND	Suspected	ND
7/A	No	No	No	ND	ND	ND	Suspected	ND
8/B	No	No	No	ND	ND	ND	Suspected	ND
9/C	No	No	No	ND	ND	ND	Suspected	ND
10/B	No	No	No	ND	ND	ND	Suspected	ND
11/B	No	No	No	ND	ND	ND	Suspected	ND
12/A	Yes		Serum/sputum	+	+†	–	Confirmed	+
13/A	Yes		Serum/sputum	+	+†	–	Confirmed	+
14/B	No	No	No	ND	ND	ND	Suspected	ND
15/D	Yes		Serum	ND	–	ND	Suspected	ND
16/A	Yes		Serum	ND	–	ND	Suspected	ND
17/B	Yes		Serum	ND	+†	ND	Confirmed	ND
18/E	No	No	No	ND	ND	ND	Suspected	ND
19/E	No	No	No	ND	ND	ND	Suspected	ND
20/F	No	No	No	ND	ND	ND	Suspected	ND

*RDT, rapid dipstick test; WHO, World Health Organization; ND, not done; +, positive; –, negative.

†A 4-fold increase in titer in the second serum sample.

Table 2. Characteristics of 41 contacts and plague patients during pneumonic plague outbreak, northern Madagascar, 2011*

Contact/relationship	Symptoms	Chemoprophylaxis	Serologic result†	WHO case definition	Village	Characteristic
1–5, family	None	Yes	–	NA	Ankatakata	Houses A and B
6, family	None	Yes	–	NA	Antsakoabe	House C, husband of patient 9
7, family	None	Yes	–	NA	Antsakoabe	House C, children of patient 9
8 and 9, family	None	Yes	–	NA	Antsakoabe	House C
10, family	None	No	–	NA	Tsatanana	House D
11–13, family	None	Yes	–	NA	Ankiganyo	House E
14, family	None	Yes	+	Presumptive	Ankiganyo	House E, mother of patient 18
15 and 16, family	None	Yes	–	NA	Ambarakaraka	House F
17–24, other	None	Yes	–	NA	Mandrivary	Met with patient 10
25, other	Cough	No	+	Presumptive	Mandrivary	Carried patient 3
26, other	None	Yes	–	NA	Antafiabe	Shared bed with patient 11
27–31, other	None	Yes	–	NA	Antafiabe	Met with patient 11
32–34, other	Mild	Yes	–	NA	Several	Attended funerals
35–41, other	None	Yes	–	NA	Several	Attended funerals

*Contacts of plague patients were divided into family contacts (persons who lived in the same household with an infected person) and other contacts (persons spent time with a patient or approached a persons who died). WHO, World Health Organization.

†For *Yersinia pestis*.

(CRISPRs) were identified (21–23). Sequences obtained were compared with sequences reported (21–23) and those in the CRISPR database (<http://crispr.u-psud.fr/>). All 10 *Y. pestis*-positive samples had an identical *Ypa* locus (a1-a2-a3-a4-a5-a6-a7-a8). For the *Ypb* locus, 1 spacer reduction (b5) was detected in a sample from case-patient 13. The other 9 samples showed an identical *Ypb* locus (b1-b2-b3-b4-b5). Seven samples had an identical *Ypc* locus (c1-c2-c3). However, within this locus, a new, and to our knowledge, Madagascar-specific spacer c12 was detected in *R. rattus* rodent sample R16 from Ambarakaraka and in a Bealanana-013 sample from 2010 (Table 4). The newly found spacer sequence originated from a phage: c12: 5'-ATCGAGGCGGGCCGGAAGAATGTCACGGCG-GTT-3' (by BLAST analysis; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The presence of spacer c12 shows a correlation

with a 1-nt reduction in the precedent spacer (c3>c3') in both samples (Table 4). Despite performing several PCRs, we could not amplify the *Ypc* locus from *R. rattus* rodent sample R05 from Antanabe (Table 4).

Discussion

Three plague pandemics and numerous plague epidemics have been caused by *Y. pestis* in the past 1,500 years (2,4,7,12,24). During the second medieval pandemic, which also included the Black Death period, 50% (~50 million persons) of the human population in Europe reportedly died of plague (2,4). At the beginning of the 20th century, 10,000 persons died during 2 plague epidemics in Manchuria, China (12). Although plague is still endemic to other countries, the reported numbers of plague patients has decreased to an average of 4,000/year

Table 3. Analysis of animal serum samples and organs for *Yersinia pestis*, April 1, 2011, during pneumonic plague outbreak, northern Madagascar, 2011*

Village	Rodent or dog source	No. samples	Serologic result	<i>pla/caf1</i> PCR result	Culture	SNPs	CRISPR	Sample ID
Antanabe	<i>Rattus rattus</i>	1	–	+	–	+	+	R05
	<i>R. rattus</i>	20	–	–	ND	ND	ND	NA
	<i>Mus musculus</i>	1	–	–	ND	ND	ND	NA
	<i>Suncus murinus</i>	1	–	–	ND	ND	ND	NA
Ambarakaraka	<i>R. rattus</i>	1	–	+	–	+	+	R16
Ankatakata	<i>R. rattus</i>	1	–	+	–	+	+	R48
	<i>R. rattus</i>	1	–	+	–	+	+	R52
	<i>R. rattus</i>	1	–	+	–	+	+	R56
	<i>R. rattus</i>	15	–	–	ND	ND	ND	NA
	<i>Canis sp.</i>	2	+	ND	ND	ND	ND	NA
	<i>Canis sp.</i>	3	–	ND	ND	ND	ND	NA
	<i>M. musculus</i>	2	–	–	ND	ND	ND	NA
<i>Setifer setosus</i>	1	+	–	ND	ND	ND	NA	
Antsisoboko	<i>R. rattus</i>	2	–	–	ND	ND	ND	NA
	<i>S. setosus</i>	2	–	–	ND	ND	ND	NA
	<i>Microgale breviceaudata</i>	3	–	–	ND	ND	ND	NA
Antanambao	<i>R. rattus</i>	9	–	–	ND	ND	ND	NA
	<i>S. setosus</i>	3	–	–	ND	ND	ND	NA
Total	NA	69	3 positive	5 positive	NA	5	5	NA

**pla*, plasminogen activator gene; *caf1*, capsule antigen fraction1 gene; SNPs, single-nucleotide polymorphisms; CRISPR, clustered regularly interspaced short palindromic repeat pattern; ID, identification; –, negative; +, positive; ND, not done; NA, not applicable.

Table 4. Molecular typing of *Yersinia pestis* in 10 samples from humans and rats during pneumonic plague outbreak, northern Madagascar, 2011*

Sample origin	Source†	Distance, km‡	Year	<i>Ypb</i> locus	<i>Ypc</i> locus§
Ankatakata, case 12	Human	0	2011	b1-b2-b3-b4-b5	c1-c2-c3
Ankatakata, case 13	Human	0	2011	b1-b2-b3-b4	c1-c2-c3
Ankatakata #R48	Rat	0	2011	b1-b2-b3-b4-b5	c1-c2-c3
Ankatakata #R52	Rat	0	2011	b1-b2-b3-b4-b5	c1-c2-c3
Ankatakata #R56	Rat	0	2011	b1-b2-b3-b4-b5	c1-c2-c3
Antanabe #R05	Rat	3	2011	b1-b2-b3-b4-b5	NA
Ambarakaraka #R16	Rat	3	2011	b1-b2-b3-b4-b5	c1-c2-c3'-c12
Bealanana #013	Human	400	2010	b1-b2-b3-b4-b5	c1-c2-c3'-c12
Ankazobe #275	Human	400	2010	b1-b2-b3-b4-b5	c1-c2-c3
Mandritsara #438	Human	800	2011	b1-b2-b3-b4-b5	c1-c2-c3

*The s232 locus for all 10 isolates had a derived status, and the 13 single-nucleotide polymorphisms (s1362, s1375, s190, s197, s1367, s1004, s1025, s1377, s1089, s1363, s206, s1373, and s152) for all 10 isolates had an ancestral status.

†Rat, *Rattus rattus*.

‡Distance to outbreak village (Ankatakata).

§c3, 5'-CTGAAATACAAATAAAATAAATCGTTCGAACAT-3'; NA, no amplification; c3'-CTGAAATACAAATAAAATAAATCGTTCGAACA-3'; c12, 5'-ATCGAGCGGGCCGGAAGAATGTCACGGCGGTT-3'.

since 1954. Also, there has been a slight decrease regarding the highest reported case number from countries in Asia and Africa (8,9).

Since the introduction of plague to Madagascar in 1898, the Institute Pasteur in Antananarivo was assigned to control this disease. The pneumonic plague outbreak in 2011 shows that despite introduction of education programs, a plan to investigate plague outbreaks promptly, and a trained task force, plague outbreaks cannot be prevented. However, in contrast to control of historical plague, when treatment with antimicrobial drugs was not available and the disease could spread unhindered, the present outbreak was stopped quickly after 27 days because of successful treatment with antimicrobial drugs (Figure 2). The good response of the patients to these drugs suggests that the *Y. pestis* strain that caused this outbreak was susceptible to streptomycin. However, streptomycin-resistant *Y. pestis* strains were isolated in Madagascar in 1995 and also during the outbreak in 2011 (25; M. Rajerison, pers. comm.). Therefore, drug-resistant *Y. pestis* strains may pose a new challenge to health authorities.

During the outbreak in 2011, persons shared single-room houses, lived in extended families, and closely cared for each other. Patients were not isolated. Because of social conventions, some persons trusted a traditional healer rather than physicians. This finding led to the third wave of the outbreak, which included 3 case-patients with pneumonic plague who died (Figure 2; Table 1).

WHO lists plague as a disease for which patients should be quarantined and requires that pneumonic plague patients are isolated from healthy persons (9). However, during the latency period before hemoptysis, sputum contains hardly any infectious organisms (11,26). Simple countermeasures, such as protective facial masks, are efficient in preventing transmission by droplets. Also, turning one's head away from or turning one's back toward a healthy person has a major prophylactic effect (26). This finding might explain why c26, who shared the

same bed with case-patient 11 until his death, was not infected (Tables 1, 2). Thus, knowledge of the pathogenesis of *Y. pestis* in humans is essential for persons who live in plague-endemic countries.

It has been suggested that patients with bubonic plague and patients who have died of plague are not directly infectious to other humans (9,26). This suggestion is consistent with findings in the present study because contacts (c32–c41) who only attended the funerals did not show symptoms or seroconversion (Table 2; Figure 2).

Plague is endemic to Madagascar, especially in the central highlands (7). At an altitude >800 m, large numbers of rodent species and insectivores live in the rain forest. Those animals represent the classical natural focus for *Y. pestis* (7). The low-elevation seaport villages of Mahajanga and Antananarivo are exceptions to this altitude factor. Because of trade and stockpiling of grain and other food products, homophilous species, such as the black rat (*R. rattus*) and the Norwegian rat (*R. norvegicus*), play a major role in the urban lifecycle of plague (6,7,27). The present outbreak occurred at an altitude <500 m in a region that does not have much commercial or economic activity. Because of this finding, there was a low prevalence of small mammals in this area, and only a low number of rodents and insectivores were trapped during the epidemiologic investigation (Table 3). This factor resulted in the plague outbreak not being immediately recognized.

Although the outbreak *Y. pestis* strain could not be isolated, information was obtained by molecular analyses of human and animal samples. All samples contained the Madagascar-specific 1.ORI3-k genotype of *Y. pestis*, as previously reported (1,6). Results of CRISPR typing identified >1 genotype, which indicated that the outbreak area was a natural plague focus before the outbreak in 2011 (Table 4). This result is supported by an unusual high prevalence (12%, 8/69) of *Y. pestis*-positive animal samples (Table 3) compared with prevalences in previous studies (28–30). We suggest that *Y. pestis* strains

containing the major CRISPR profile, which was found in 6 of 10 samples, was responsible for the present outbreak (Table 4). This CRISPR genotype has also been found in samples from the central highlands. The isolate from case-patient 13 lost the b5 spacer (Table 4). This phenomenon has been reported for other CRISPR profiles, and a different genotype has been assessed (21–23). A third *Y. pestis* CRISPR genotype was found in the *R. rattus* rodent R16 sample and in the human Bealanana 013 sample from 2010; this genotype includes the new element c3'–c12 of phage origin (Table 4) (21). The loss of 1 nt at the end of the spacer, as observed in c3>c3', has been previously reported (22).

Despite performing several PCRs, we could not amplify the complete *Ypc* locus in the *R. rattus* rodent R05 sample from Antanabe (Table 4), a finding that has been previously reported (21). We suggest that 4 CRISPR genotypes of *Y. pestis* were present in the outbreak area, which indicates that Ambilobe was a natural plague focus even before the outbreak. Unnoticed presence of pathogens near human populations requires higher surveillance activity, as recently reported (7). In contrast, it has been reported that hereditary resistance against *Y. pestis* might develop in rats (7,31).

One question that also needs to be addressed is why plague caused millions of deaths during devastating pandemics in the past while today plague is restricted to some geographic locations. One possible explanation would be the presence of additional virulence factors in historical plague strains, which have been lost from current *Y. pestis* strains.

To answer this question, 2 ancient *Y. pestis* genomes were sequenced and compared with sequences of current *Y. pestis* isolates. The ancient genomes were from a pandemic European *Y. pestis pestis* biovar Antiqua isolate (genotype 1.ANT) that originated during the Black Death period (3), and from a pandemic *Y. pestis pestis* biovar Antiqua isolate (genotype 0.ANT), which was isolated from a patient who died during the plague of Justinian in Germany (5). Sequences of current *Y. pestis* genomes used for comparison were from the nonhuman pathogenic Chinese *Y. pestis microtus* strain (91001: 0.PE4, biovar Xilingolensis) and from the *Y. pestis pestis* strain (CO92: 1.OR11, biovar Orientalis) (3,5).

Analyses showed that genomes of ancient *Y. pestis* strains did not contain additional virulence genes that might explain higher virulence. (3,5). Furthermore, because plague epidemics were caused by different biovars or genotypes in Asia, Europe, and Africa (3,5,6,10,12–15), we assume that various *Y. pestis* subtypes are similar in virulence. Thus, factors other than differences in virulence might better explain the decrease in the reported plague cases. It is more likely that the general perception and understanding of infectious agents; improved hygiene; trade management; knowledge of plague pathogenesis in humans; vector control;

specific outbreak management, including selective isolation of infectious patients; and chemoprophylaxis and treatment with antimicrobial drugs, have accounted for the decrease in plague cases (9,11,15,26).

In conclusion, the 1.OR13-k genotype of *Y. pestis* identified in the present study has virulence comparable with that of ancestral genotypes that caused other epidemics. The course and outcome of a human plague epidemic depend on this virulence, as well as on education, public awareness, life style, infrastructure, isolation of patients, and medical care. However, there is an unpredictable pathogenic potential in drug-resistant strains that has not been estimated and needs to be studied.

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Epidemiology of Human Plague in the United States, 1900–2012

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Learning Objectives

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

- Analyze the broad epidemiology of human plague in the United States
- Identify the most common primary clinical form of human plague in the United States
- Evaluate temporal trends in the epidemiology of human plague
- Assess survival outcomes of human plague in the United States.

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We summarize the characteristics of 1,006 cases of human plague occurring in the United States over 113 years, beginning with the first documented case in 1900. Three distinct eras can be identified on the basis of the frequency, nature, and geographic distribution of cases. During 1900–1925, outbreaks were common but were restricted to populous port cities. During 1926–1964, the geographic

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range of disease expanded rapidly, while the total number of reported cases fell. During 1965–2012, sporadic cases occurred annually, primarily in the rural Southwest. Clinical and demographic features of human illness have shifted over time as the disease has moved from crowded cities to the rural West. These shifts reflect changes in the populations at risk, the advent of antibiotics, and improved detection of more clinically indistinct forms of infection. Overall, the emergence of human plague in the United States parallels observed patterns of introduction of exotic plants and animals.

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Plague is a globally distributed, zoonotic disease caused by the bacterium *Yersinia pestis* (1,2). In the late 1890s, rat-infested steamships introduced the disease into the continental United States (1,3,4). The first documented autochthonous human infection occurred in the Chinatown section of San Francisco, California, in March of 1900. Cases were soon reported in other port cities, including New Orleans, Galveston, Seattle, and Los Angeles (3,5). Along the Pacific Coast, infection spread from urban rats to native rodent species, and by the 1950s, *Y. pestis* had spread eastward to reach western portions of the Dakotas, Nebraska, Kansas, Oklahoma, and Texas. This distribution has remained static for >60 years, presumably the result of climatic and ecologic factors that limit further spread (3,5–9). Although poorly defined, these factors may be related to the ecology of vector species rather than that of rodent hosts (8).

The history of human plague in the United States provides a unique opportunity for long-term study of a zoonotic disease introduced onto a continent. Although the medical and scientific literature has detailed case histories and epidemiologic findings of plague cases in the United States, most reports have been limited in geographic scope or time frame (4–6,10–21). We use data from all reported human plague cases in the United States during 1900–2012 to summarize and describe changes in the epidemiology of plague since its introduction.

Methods

The basis for plague diagnosis has changed over the last century. For purposes of this summary, a case of plague was defined as a clinically compatible human illness and at least 1 of the following: 1) *Y. pestis* isolated from or detected in a clinical specimen, 2) elevated antibody titer to *Y. pestis* F1 antigen in ≥ 1 serum specimen (22), or 3) supportive epidemiologic and other laboratory evidence (e.g., visualization of typical *Y. pestis* morphology on a stained slide). The clinical form of plague (e.g., bubonic, pneumonic, septicemic) was determined on the basis of explicit notations in the case records or from available clinical details; only the primary clinical form was considered. For example, patients who had primary bubonic plague and secondary pneumonic plague were classified as having bubonic plague.

Data from cases occurring during 1900–1981 were collected from lists maintained by the United States Public Health Service and later the US Centers for Disease Control and Prevention (CDC), and enhanced with additional data sources including state reports and publications in the peer-reviewed literature. Supplementary detailed information on clinical course, exposure, and treatment was collected and maintained for most plague patients beginning in 1956, continuing through the present time.

Human cases acquired within the continental United States were included in this summary. Case-patients were geographically represented by state of residence or state of exposure, as indicated. Antibiotics considered effective for plague for the purpose of this analysis were: streptomycin, gentamicin, tetracycline/doxycycline, chloramphenicol, fluoroquinolones, or co-trimoxazole (22). Categorical variables are described as counts and proportions, and statistically compared by using χ^2 tests with $\alpha = 0.05$. Continuous variables are described by median and range.

Results

Descriptive Epidemiology

A total of 1,006 human plague cases were reported during 1900–2012. Infections were acquired in 18 states among residents of 20 states. Median patient age was 29 years (range <1–94 years), and 644 (65%) of the 992 patients for whom sex was reported were male. Among 913 cases with documented primary clinical form, 744 (82%) were bubonic, 74 (8%) pneumonic, 87 (10%) septicemic, 6 (1%) pharyngeal, and 2 (<1%) gastrointestinal. White non-Hispanic persons accounted for 55% of all cases when race or ethnicity was known. American Indian and Asian persons each accounted for 16%. Persons identified as Hispanic comprised 12% of cases; for 20%, race or ethnicity were unknown. Overall patterns of disease frequency and geographic distribution suggest 3 distinct epidemiologic phases over the course of the 113-year period. These phases correspond roughly to the periods 1900–1925, 1926–1964, and 1965–2012 (Figure).

During 1900–1925, 496 plague cases were reported (median 3.5 cases per year), accounting for roughly half of all human plague cases in the United States during the 113 years surveyed (Table 1). Cases were restricted almost exclusively to port cities on the Pacific and Gulf coasts; 90% occurred in California and Louisiana (Figure). Variation among years was pronounced: 191 cases were recorded in 1907, and 0 were recorded in 5 (19%) of the 26 years (Table 1, Figure) in this period. The median age of patients was 30 years (range <1–84 years): 71% were male, and >30% were identified as Asian (Table 1). This period included several outbreaks of pneumonic plague characterized by person-to-person transmission; the last of these occurred in 1924 in Los Angeles. Consequently, pneumonic cases were more common (15%) than in later periods (Table 1). The seasonal distribution of cases peaked in September.

The second period, 1926 to 1964, accounted for only 42 (4%) of the 1,006 cases, a median of 1 case per year (Table 1). Nevertheless, the rare cases were distributed inland, and except for 1 laboratory-acquired case in Maryland, were acquired in multiple western states

(Figure). Overall, 52% (22/42) of cases occurred in California, 29% (12/42) in New Mexico, and 1 or 2 cases each in Arizona, Colorado, Idaho, Oregon, and Utah. No more than 5 cases were reported in any single year (Table 1; Figure), and no cases were reported in 17 (44%) of the 38 years in this second period. The median age was markedly lower (15 years; range 3–67 years), more patients were male (83%), and more were non-Hispanic Whites (83%), as compared to other periods (Table 1). The median disease onset was in July, which was 2 months earlier than for 1900–1925.

There were 468 (47%) reported plague cases during the period of 1965–2012 (Table 1). A median of 8 cases occurred per year, and unlike previous periods, cases occurred every year for the entire 48 years (Table 1; Figure). The majority of cases occurred in the Southwest; 54% (251/467) of patients were infected in New Mexico, 14% (63/467) in Arizona, 12% (56/467) in Colorado, and

9% (42/467) in California (Figure). The median age of case-patients was 28 years (range <1–94 years). Males accounted for a lower proportion (57%) and American Indian persons represented a much larger proportion of cases (33%) than during previous years. Primary septicemic disease was more common than during earlier periods, accounting for 17% of all cases (Table 1). The median disease onset was July, the same seasonal distribution as the previous time period.

Clinical Features and Outcome

The first documented use of antibiotics to treat plague in the United States was in 1942. Among 511 plague cases occurring before 1942 with outcome information, 336 (66%) were fatal, with similar case-fatality rates for male and female patients (Table 2). Mortality rates were highest among patients with septicemic (89%) and pneumonic (93%) forms of infection (Table 2). In addition,

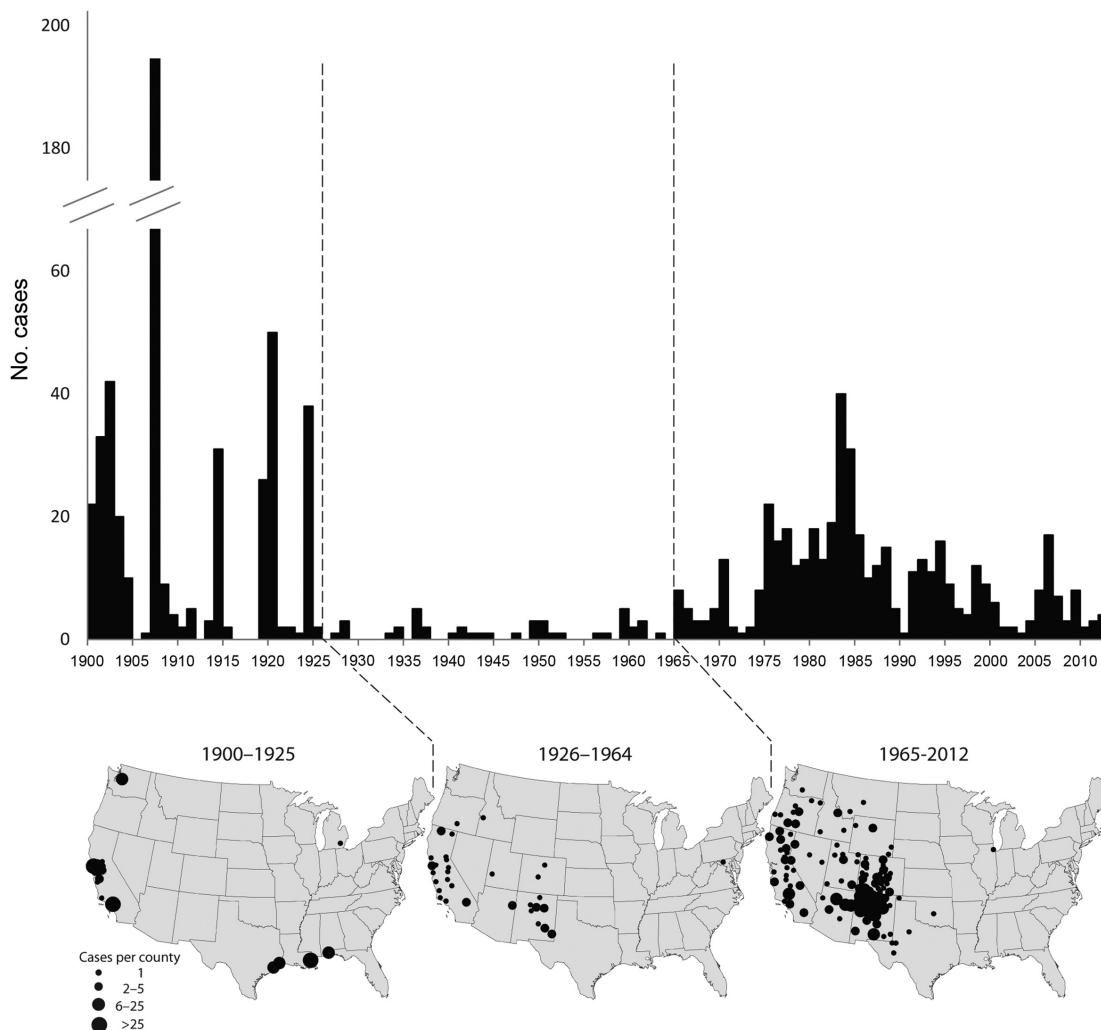


Figure. Frequency and geographic distribution of human plague cases in the United States, 1900–2012. Three periods reflect different epidemiologic and geographic patterns: 1900–1925, 1926–1964, and 1965–2012.

Table 1. Epidemiologic characteristics of human plague, United States, 1900–2012*

Characteristic	1900–1925	1926–1964	1965–2012	All years
No. cases	496	42	468	1,006
Median no. cases per year (range)	3.5 (0–191)	1 (0–5)	8 (1–40)	3 (0–191)
No. counties with reported case exposures	18	32	113	135
Years with no reported cases, %	19	44	0	20
Male sex	341/483 (71)	35/42 (83)	268/467 (57)	644/992 (65)
Median age, y (range)	30 (<1–84)	15 (3–67)	28 (<1–94)	29 (<1–94)
Race/ethnicity				
White	224/409 (55)	19/23 (83)	198/370 (54)	441/802 (55)
Asian	127/409 (31)	0/23 (0)	3/370 (1)	130/802 (16)
American Indian	1/409 (<1)	3/23 (13)	123/370 (33)	127/802 (16)
Hispanic	46/409 (11)	1/23 (4)	46/370 (12)	93/802 (12)
Black	11/409 (3)	0/23	0/370	11/802 (1)
Primary clinical form				
Bubonic	344/415 (83)	31/36 (86)	369/462 (80)	744/913 (82)
Pneumonic	60/415 (15)	3/36 (8)	11/462 (2)	74/913 (8)
Septicemic	8/415 (2)	2/36 (6)	77/462 (17)	87/913 (10)
Pharyngeal	3/415 (<1)	0/36 (0)	3/462 (1)	6/913 (1)
Gastrointestinal	0/415 (0)	0/36 (0)	2/462 (<1)	2/913 (<1)
Route of infection, no. (%)†				
Person-to-person	49 (10)	0	0	49 (5)
Known flea bite	2 (<1)	3 (7)	101 (22)	106 (11)
Animal butchering/skinning	6 (1)	8 (19)	50 (11)	64 (6)
Animal bite/scratch/cough	2 (<1)	0	19 (4)	21 (2)
Animal handling	0 (0)	5 (12)	86 (18)	91 (9)
Unknown	437 (88)	26 (62)	238 (51)	701 (70)

*Values are no. assessed/no. cases (%) except as indicated.

†During 1965–2012, a total of 26 case-patients had both known flea bite and animal contact.

the mortality rate during this time was higher among racial minorities (91% overall) than among non-Hispanic Whites (53%) (Table 2).

After the introduction of antibiotics, the overall proportion of plague infections that resulted in death decreased substantially, from 66% to 16% (Table 2), albeit incrementally. During 1942 through 1964, 44% (11/25) of cases were fatal; after 1965, the mortality rate remained stable at ≈13%. Lower mortality was specifically seen in those patients who received effective antibiotics. Among 433 cases with treatment and outcome information, only 34 (9%) of 377 case-patients who received at least 1 dose of an effective antibiotic died, compared with 29 (52%) of 56 who received either no treatment or ineffective treatment. Primary bubonic plague case-patients more often received effective antibiotics, although the difference was not substantial (bubonic 89%; septicemic 81%; pneumonic 79%). The magnitude of decrease in deaths was similar for most clinical forms; however, the proportion of pneumonic plague cases that were fatal (36%) remained ≈3-fold higher, and of septicemic plague, ≈2-fold higher (27%), than the proportion of bubonic plague cases that were fatal (13%). Notably, although there were few cases (989), 67% of pharyngeal plague case-patients died, regardless of availability of effective treatment. After the introduction of antibiotics, the overall mortality rate did not differ with patient age or race, but was higher for male patients than for female patients (19% versus 11%, respectively; Table 2).

Sources of Infection

Information on route of exposure to *Y. pestis* was documented for only 30% of cases. Of the 305 persons for whom specific exposure information was available, 106 patients had a known flea (or “insect”) bite, 91 had recently handled an animal, 64 had butchered or skinned an animal, and 21 had reported an animal bite, scratch, or cough (Table 1). Twenty-six patients who had flea bites also had a record of animal contact. Forty-nine cases occurred as the result of person-to-person transmission, most during pneumonic plague outbreaks that occurred in crowded, urban settings in the early 1900s (Table 1). Of the remaining plague cases without exposure information, an additional 139 case-patients had documented buboes in the inguinal or femoral region, a clinical finding suggestive of flea exposure (20).

Among case-patients who had a known flea bite, 95 cases (90%) were primary bubonic plague and 10 (9%) were primary septicemic plague. Of the 89 case-patients who had flea-acquired bubonic plague and bubo information, 59 (66%) displayed either inguinal or femoral adenopathy. Among case-patients with a history of animal contact, most cases had primary bubonic plague, but the proportion varied by type of animal contact (91% for butchering or skinning; 77% for handling; and 71% for an animal bite, scratch, or cough). Most of the case-patients with a history of an animal bite, scratch, or cough (16/21, 76%) were exposed to domestic cats. Additionally, in 6 (43%) of the 14 primary pneumonic cases that had occurred since 1924 (the

last documented case of person-to-person transmission), case-patients had contact with domestic cats.

During the antibiotic era beginning in 1942, the mortality rate among plague case-patients with a history of animal contact (36/173, 21%) was higher than among those with only a recognized flea bite (7/80, 9%) (χ^2 test, $p = 0.018$). Notably, this difference in survival was not related to receipt of effective antibiotics. Specifically, neither history of flea bite nor of animal contact was associated with receipt of effective antibiotics (χ^2 test, $p = 0.848$, $p = 0.499$, respectively).

Records for 27 case-patients indicated a specific exposure associated with the patient’s occupation, including the only known, nonimported plague cases to occur in the eastern United States outside of port cities (i.e., Michigan, Maryland, and Illinois) (Figure). Cases occurred in the following occupational groups: 8 (30%) veterinarians, 5 (19%) persons who worked with animals (e.g., wildlife biologist or animal control personnel), 5 (19%) plague laboratory researchers, and 3 (11%) persons who conducted autopsies during the early 1900s. Since 1924, 3 (21%) of the 14 primary pneumonic plague cases occurred in persons conducting laboratory or primate research on plague. Remaining occupation-associated exposures were less direct (e.g., a geologist on a research trip, a camp counselor).

Discussion

Invasion of a geographical location by exotic plant and animal species has been described as a multistep process involving transport, introduction, establishment, spread, and progressive impact on the ecology and human population (23–25). Multiple introductions are often necessary for an invading species to become established, and once established, a “lag period” of years to decades often ensues during which the invading species remains relatively localized. This is typically followed by a period of rapid geographic spread, ultimately resulting in increased ecologic and human effects.

Seen from the perspective of human infection, the emergence of plague in the United States is analogous to the invasion process of exotic plant and animal species. Available evidence indicates that *Y. pestis* was introduced on multiple occasions into various port cities; however, establishment appears to have been successful only in Pacific port cities (3,7). In San Francisco, infection spread from a cycle involving urban rats and their fleas into native wild ground squirrels (*Citellus* spp.) on the outskirts of the city by 1908 (3). In contrast, *Y. pestis* apparently never successfully established in wild rodent populations outside of Gulf coast port cities, likely a result of inhospitable ecology and early and extensive urban rat control efforts (3,5,8).

For the next 2 decades, the disease remained localized to port cities and surrounding areas, causing intermittent

Table 2. Mortality among human plague patients in the preantibiotic (1900–1941) and antibiotic (1942–2012) eras, United States

Characteristic	No. deaths/no. cases (%)	
	1900–1941	1942–2012
Total per period	336/511 (66)	75/478 (16)
Sex		
M	238/353 (67)	53/282 (19)
F	93/145 (64)	22/195 (11)
Race/ethnicity		
White	123/231 (53)	34/205 (17)
Asian	121/127 (95)	1/3 (33)
American Indian	1/1 (100)	19/122 (16)
Hispanic	38/47 (81)	6/44 (14)
Black	10/11 (91)	0
Primary clinical form		
Bubonic	235/354 (66)	47/375 (13)
Pneumonic	55/59 (93)	5/14 (36)
Septicemic	8/9 (89)	21/78 (27)
Pharyngeal	2/3 (67)	2/3 (67)
Gastrointestinal	0	0/2

but large outbreaks. Following this lag period of minimal spread, the pathogen began dispersing rapidly eastward from the Pacific Coast (7). In 1935, *Y. pestis* was documented among wild rodents outside of California. By the early 1950s, plague had reached its current geographic distribution, and since 1965 has caused consistent human illness, suggesting that *Y. pestis* has become fully entrenched in enzootic cycles throughout the West (3). Together with their respective flea species, multiple rodents contribute to the current ecology of plague in United States, including ground squirrels, prairie dogs, wood rats, chipmunks, deer mice, and voles (26).

Shifts in epidemiologic features of human plague have occurred during the years following its introduction. The demographic characteristics of plague patients are inextricably linked to the geographic distribution of the organism over time and the populations at risk in those areas. For example, the disproportionate excess of men among Asian case-patients in the early years may solely represent the male-dominated Asian immigrant population in the port cities of the Pacific at the beginning of the 20th century (27,28). The demographic shift in burden of illness from persons of Asian heritage to those of American Indian heritage over time reflects change in the geographic distribution of plague from overcrowded urban neighborhoods dominated by immigrant populations along the Pacific Coast to the rural areas in the Southwest, including tribal lands. The proportional increase over time in primary septicemic cases was likely the result of better recognition of a clinically indistinct form of infection coincident with improved laboratory diagnostic capability (i.e., blood cultures), or possibly a increase in number of infections associated with direct animal contact.

The overall plague mortality rate decreased with availability of effective treatment. Nevertheless, the risk of

death from plague infection is still substantial, particularly for patients with primary septicemic, pneumonic, and pharyngeal manifestations. Although exposure information was limited, infection acquired through animal contact was associated with higher mortality than was infection transmitted by flea bite. Receipt of effective antibiotics was similar between case-patients who had animal contact and those bitten by fleas. The difference in mortality rates may be related to higher or more direct initial inoculum of bacteria or differences in protein expression between mammals and fleas (29–31).

This summary of human plague in the United States may underrepresent some infections. Although *Y. pestis* is among the most pathogenic bacteria known, and mild, self-limiting infection is not considered commonplace, this summary could exclude mild and undiagnosed infections (32). Unrecorded plague infections may also have occurred with unrecognized septicemic disease or among racial or ethnic minorities, whose populations typically have less access to medical care. Additionally, minorities may not have sought medical care because of fear of racial prejudice associated with the initial introduction of plague (3,33). Unrecorded plague infections would more likely have occurred in the early 20th century, however, and are unlikely to dramatically affect the overall trends observed in this analysis.

Now an endemic zoonosis in the United States, plague is likely to continue causing rare but severe human illness in western states. Historically, plague was often linked to poor sanitation that resulted in rodent infestations. However, plague in New Mexico has increasingly occurred in more affluent areas, a result of continued suburban and ex-urban development in enzootic plague foci (11,36). Regardless of a person's race, ethnicity, or socioeconomic status, the primary risk factors for plague infection in the United States are behaviors and conditions that increase both direct and indirect human contact with rodents and their fleas. In recent years, few patients have reported clear exposures to infected animals or rodent fleas; most were likely infected while performing common outdoor peridomestic work (e.g., cutting brush or chopping wood) or as a result of contact with infected fleas that were brought into the home by indoor/outdoor pets (18,37; CDC, unpub. data). Clinical suspicion remains critical to early and appropriate treatment. Recommended treatment is with aminoglycosides and tetracyclines, but fluoroquinolones may also be effective (34). The US Food and Drug Administration recently approved levofloxacin for treatment of patients with plague, based on in vitro and animal studies (35). Additional antibiotics considered effective for the purposes of this summary should not be considered first-line treatment for plague. Accurate plague diagnosis is further challenged by reliance on automated identification systems that frequently

misidentify *Y. pestis* (38). A few travel-associated cases have been diagnosed in areas without endemic plague (e.g., South Carolina, New York City, Connecticut), highlighting the importance of plague in the differential diagnosis of ill persons, even those lacking apparent lymphadenopathy, with recent travel history anywhere in the western United States (39,40; CDC, unpub. data).

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Health Care Response to CCHF in US Soldier and Nosocomial Transmission to Health Care Providers, Germany, 2009¹

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In 2009, a lethal case of Crimean–Congo hemorrhagic fever (CCHF), acquired by a US soldier in Afghanistan, was treated at a medical center in Germany and resulted in nosocomial transmission to 2 health care providers (HCPs). After his arrival at the medical center (day 6 of illness) by aeromedical evacuation, the patient required repetitive bronchoscopies to control severe pulmonary hemorrhage and renal and hepatic dialysis for hepatorenal failure. After showing clinical improvement, the patient died suddenly on day 11 of illness from cerebellar tonsil herniation caused by cerebral/cerebellar edema. The 2 infected HCPs were among 16 HCPs who received ribavirin postexposure prophylaxis. The infected HCPs had mild or no CCHF symptoms. Transmission may have occurred during bag-valve-mask ventilation, breaches in personal protective equipment during resuscitations, or bronchoscopies generating infectious aerosols. This case highlights the critical care and infection control challenges presented by severe CCHF cases, including the need for experience with ribavirin treatment and postexposure prophylaxis.

Crimean–Congo hemorrhagic fever (CCHF) is a life-threatening viral illness endemic to areas of Africa, southeastern Europe, Russia, China, India, and the Middle East. CCHF is caused by infection with a tickborne virus (family *Bunyaviridae*, genus *Nairovirus*), and is generally acquired through the bite of an infected tick or contact with

blood or body fluids of infected animals (1–3). The disease is characterized by the abrupt onset of a febrile illness usually 2–7 d (range 2–14) after exposure to the virus and by subsequent severe changes in mental status, hemorrhagic manifestations, and hepatorenal failure (1,4). Case-fatality rates vary by region but are 30%–50% (range 1%–73%) in most regions; death generally occurs 5–14 d after symptom onset and is most commonly a result of multi-organ failure, shock, severe anemia, cerebral hemorrhage, and/or pulmonary edema (1,5).

We report a fatal case of CCHF in a US soldier deployed to Afghanistan, who was aero-evacuated to Germany for treatment, and the documented nosocomial infection of 2 health care providers (HCPs) who were at risk for exposure and had received ribavirin postexposure prophylaxis (PEP). We also review infection control interventions and contact surveillance, both of which were required because of the patient's severe bleeding and the risk for aerosol production. Research on human subjects was conducted in compliance with US Department of Defense, federal, and state statutes and regulations relating to the protection of human subjects and adheres to the principles identified in the Belmont Report (1979; <http://www.hhs.gov/ohrp/humansubjects/guidance/belmont.html>).

The Case

On September 8, 2009, a 22-year-old male US soldier who worked in field operations outside Kandahar City, Afghanistan, sought care at a military medical clinic for a 4-d history of nonbloody diarrhea, abdominal pain, bloody emesis, and fever (39.2°C). The patient reported frequent outdoor activities,

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tick bites, and exposure to undercooked goat meat and blood the week before the onset of illness. Ciprofloxacin was prescribed for probable gastroenteritis. The patient did not improve by the next day and returned to the clinic, reporting somnolence and lethargy. He was transferred to a Combat Support Hospital at Kandahar Air Base.

Admission laboratory values demonstrated anemia, thrombocytopenia, acute renal insufficiency, and elevated levels of hepatic transaminases. Within a few hours of admission, the patient had worsening lethargy; bloody diarrhea; gingival bleeding; hypoxia requiring intubation; and a hypotensive episode after intubation, which required vasopressor therapy for 24 h. The patient was treated with intravenous levofloxacin (replaced ciprofloxacin), meropenem, fresh-frozen plasma (6 U), platelets (2 U), packed erythrocytes (2 U), and infusions of furosemide and pantoprazole.

On September 10, the patient was emergently aeroevacuated to Landstuhl Regional Medical Center (LRMC; Landstuhl, Germany). During the flight, his respiratory status deteriorated (requiring 100% FiO₂) and he continued to bleed from multiple sites (nares, gingiva, gastrointestinal, and venipuncture sites). Treatment on route included fresh-frozen plasma (6 U), packed erythrocytes (1 U), and cold-water lavage to decrease upper gastrointestinal bleeding.

The patient arrived at LRMC on September 11 (day 6 of illness); he was in multiorgan, failure and large amounts of bright red blood were in the endotracheal tube. During the initial physical examination, he exhibited respiratory compromise, temperature of 37.3°C, blood pressure of 147/74 mm Hg (reference values for clinical/laboratory values are in Table 1), pulse rate of 118 beats/min, and 90% saturation on 100% oxygen. Significant findings included edematous conjunctivae with mild hemorrhage; nasopharyngeal bleeding; coarse breath sounds; large ecchymoses at venipuncture sites; scattered petechiae on the trunk, arms, and upper thighs; extensive edema of the extremities and scrotum; and melena on rectal examination. Emergent bronchoscopy revealed diffuse bleeding in the airways. Admission laboratory and radiography results (Table 1) supported a presumptive diagnosis of CCHF, and infection control measures for possible viral hemorrhagic fever (VHF) were implemented (9).

On day 7 of illness, reverse transcription PCR (RT-PCR) of a serum sample obtained at admission showed a CCHF viral load of 1.2×10^9 copies/mL (CCHF virus was later isolated from blood and urine samples obtained on day 6 of illness; CCHF IgM and IgG serologic results were negative on day 6 of illness) (Table 1). Oral and then intravenous ribavirin therapy were initiated; the intravenous ribavirin was administered within 24 h of admission and under an Investigational New Drug protocol after the patient's family gave consent (Table 1) (3,10). Treatment with the broad-spectrum antibiotics was discontinued.

Repetitive emergency bronchoscopy was required to control severe pulmonary hemorrhage.

On day 8 of illness, the patient's pulmonary status continued to deteriorate: development of adult respiratory distress syndrome required placement of bilateral chest tubes to drain bloody pleural effusions, administration of nitric oxide, and use of advanced inverse ratio bilevel mechanical ventilation. The severe adult respiratory distress syndrome was complicated by massive pulmonary hemorrhaging that required multiple bronchoscopies and infusion of blood products (packed erythrocytes [32 U], fresh frozen plasma [80 U], platelets [34 packs], factor VII [4 U], and cryoprecipitate [3 U]). Tris (hydroxymethyl) amino methane and continuous hemodialysis were initiated for progressive renal failure and severe acidosis (serum creatinine 8.2 mg/dL, bicarbonate 12 mmol/L), and multiple boluses of glucose were given for recurrent hypoglycemia.

On day 9 of illness, the patient's oxygenation and blood product transfusion requirements lessened, but hypoglycemia and acidosis persisted; a bicarbonate drip was initiated. Because fulminant hepatic failure occurred (aspartate aminotransferase 9,628 U/L, alanine aminotransferase 2,151 U/L, total bilirubin 8.1 mg/dL), liver dialysis was initiated by using a liver albumin dialysis machine. Early on day 11 of illness, liver dialysis was discontinued because the patient's condition appeared to be stabilizing (serum viral load and hepatic transaminases were decreasing, and less ventilatory support and fewer blood product transfusions were required). However, neurologic examination later that morning showed bilateral fixed and dilated pupils, and before brain imaging was possible, the patient suffered a cardiorespiratory arrest. A postmortem computer tomographic scan image demonstrated diffuse cerebral and cerebellar edema, a small right frontal parenchymal hemorrhage, and bilateral cerebellar tonsil herniation.

Hospital Infection Control

Because a CCHF diagnosis was not considered likely at the time of the initial 2 emergency bronchoscopies, standard precautions for infection control were used. The 2 bronchoscopists wore a gown, gloves, eye protection, and surgical masks; other persons in the room wore N95 respirators or surgical masks. More stringent infection control measures for VHFs, including airborne precautions, were implemented once a diagnosis of CCHF was considered (5) (Table 2). The patient was placed in an airborne-infection isolation room with an anteroom, which had restricted visitation and intensive care unit (ICU) entry. Sign-in sheets tracked who entered the patient's room; those entering were required to wear a fluid-resistant gown, gloves, N95 respirator, eye protection/face shield, and shoe coverings. Biohazard suits (Tyvek; DuPont, Richmond VA, USA) with powered air-purifying respirators were worn during subsequent bronchoscopies

Table 1. Results of laboratory testing and regimen for ribavirin treatment for a US soldier with CCHF, Germany, 2009*

Treatment/test or procedure	Day after symptom onset, date						Reference range
	Day 6, Sep 11	Day 7, Sep 12	Day 8, Sep 13	Day 9, Sep 14	Day 10, Sep 15	Day 11, Sep 16	
Ribavirin treatment†	Oral	IV	IV	IV	IV	None	NA
Test/procedure							
RT-PCR, RNA copies/mL‡	1.2 × 10 ⁹	ND	6 × 10 ⁹	ND	3 × 10 ⁸	ND	NA
Dialysis	ND	ND	Renal	Renal/hep	Renal/hep	Renal	NA
CCHF culture§	+	ND	ND	ND	ND	ND	–
IgM/IgG¶	–/–	ND	–/–	ND	+/+	ND	–
Hemoglobin, g/dL	12.8	7.7	9.1	11.9	9.9	8.5	13.2–17.1
Hematocrit, %	35.3	21.6	25.4	33.5	27.7	23.7	38–50
Leukocyte count, × 10 ³ /μL	9.6	8.8	4.9	4.0	3.4	3.5	3.5–10.5
Platelets, × 10 ³ /μL	14,000	68,000	62,000	93,000	126,000	77,000	151–356
Creatinine, mg/dL	7.8	8.7	5.1	2.7	1.4	0.9	0.8–1.5
BUN, mg/dL	67	72	32	8	2	<2	8–26
Sodium, mmol/L	140	146	143	142	141	147	137–145
Potassium, mmol/L	4.7	5.2	4.0	5.0	4.3	3.4	3.6–5.1
Bicarbonate, mmol/L	20	12	19	18	28	33	22–31
Chloride, mmol/L	110	102	98	103	100	101	101–111
Lactate, mmol/L	3.0	14.9	17.8	8.7	7.7	7.4	0.7–2.1
Glucose, mg/dL	92	187	93	68	82	89	74–106
AST, U/L	1,472	3,957	11,295	9,628	9,061	5,967	15–41
ALT, U/L	411	1,838	2,854	2,151	1,805	1,122	17–63
LDH, IU/L	756	ND	ND	ND	ND	ND	98–192
Alkaline phos, UL	186	123	163	202	254	354	38–126
Bilirubin							
Total, mg/dL	1.8	5.8	6.7	8.1	9.2	10.4	0.2–1.3
Direct, mg/dL	1.1	ND	3.3	3.2	3.0	3.0	0.1–0.3
aPTT, s	106.9	89.8	56.6	59.3	67.4	52.7	28.2–40.3
Prothrombin time, s	21.8	22.4	22.3	14.9	19.7	24	11.9–15.1
Fibrinogen, mg/dL	143	190	238	156	153	111	168–407
D-dimer, μg/dL	20	ND	ND	ND	ND	ND	<5
Albumin, g/dL	2.8	2.8	3.7	4.2	4.7	4.8	3.5–5.0
CPK, U/L	1,437	1,528	1,889	3,008	4,728	4,973	55–170
Myoglobin, ng/mL	1,226.5	ND	ND	ND	ND	ND	17.4–105.7
Other							
Malaria smear	–**	**	**	**	**	**	NA
Bacterial cultures#	–**	**	**	**	**	**	NA
Radiology							
X-ray/CT, chest	ND	Moderate to severe pulmonary edema and atelectasis	ND	ND	ND	ND	NA
CT, abdomen	ND	Ascites, gallbladder edema	ND	ND	ND	ND	NA
Cytokines							
Interleukin							
10, pg/mL ††	515	ND	1,498	ND	904	ND	<7
6, pg/mL ††	1,530	ND	>3,023	ND	2,439	ND	<15
IFN-γ, pg/mL ††	59	ND	390	ND	125	ND	<15
TNF-α, pg/mL ††	77	ND	56	ND	100	ND	<15
Growth factors							
PLGF, pg/mL ††	203	ND	64	ND	81	ND	<25
sVEGF-R1, pg/mL ††	2,930	ND	13,903	ND	13,308	ND	<180

*aPTT, activated partial thromboplastin time; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; CCHF, Crimean–Congo hemorrhagic fever; CPK, creatine phosphokinase; CT, computerized tomography; hep, hepatic; IFN-γ, interferon γ; IV, intravenously; LDH, lactate dehydrogenase; NA, not applicable; ND, not determined; phos, phosphatase; PLGF, placental growth factor; RT-PCR, reverse transcription PCR; sVEGF-R1, soluble vascular endothelial growth factor receptor 1; TNF-α, tumor necrosis factor α; –, negative; +, positive.

†On day 6, an initial 4-g loading dose (LD) of oral ribavirin was administered via nasogastric tube, followed by 1,200 mg 6 h later. On day 7, a partial LD of 22 mg/kg was administered IV (because of 60% bioavailability of oral ribavirin and poor absorption with gastrointestinal bleeding) followed by 16-mg/kg doses every 6 h (per dose-reduction protocol). Beginning day 9, 14 mg/kg was administered every 6 h, with an extension of the dosing interval to every 8 h on day 10 because of severe renal failure (only a minimal amount of drug is removed through dialysis) (3).

‡Real-time RT-PCR for virus quantification and *Nairovirus* spp.–specific gel-based RT-PCR coupled with PCR product sequencing to confirm the diagnosis (6,7).

§CCHF culture of blood and urine (virus was isolated on Vero cells and sequenced) (8).

¶CCHF-specific IgM/IgG by indirect immunofluorescence assay using CCHF virus–infected cells; assay performed at Bernard Nocht Institute, Hamburg, Germany.

#Culture of blood, urine, and sputum samples.

**Malaria smear and culture results were not specifically obtained on day 6; multiple cultures were performed.

††Testing for cytokines and vascular endothelial growth factors and their soluble receptors of blood were performed in the Biosafety Level 4 facility of Bernard Nocht Institute by using Quantikine Immunoassays (R&D Systems Europe, Abingdon, UK), according to the manufacturer's instructions.

Table 2. Isolation, infection control, PPE, and decontamination procedures used by a health care center during treatment of a US soldier infected with Crimean–Congo hemorrhagic fever virus, Germany, 2009*

Focus	Procedure
Patient	Placed in AIIR with an anteroom. Restricted visitation; sign-in sheet to track personnel entry. Entry required wearing of fluid-resistant gown and gloves (gloves pulled over edge of gown sleeve cuff), fit-tested N95 respirator, eye protection/face shield, and shoe coverings; disposal of PPE in anteroom before exiting. IPaC staff performed hands-on refresher training sessions for proper donning and doffing of PPE and for respiratory procedures (i.e., suctioning). Biohazard suits with PAPRs worn during bronchoscopies and chest tube placement.
Ventilator	Labeling of ventilators used on patient; IPaC staff–observed cleaning to ensure proper decontamination/terminal disinfection before use on another patient. Bleach 10% solution used to clean ventilators; bellows replaced; circuits discarded; internal removable parts were removed, bagged, and sterilized (viral desiccation).
Bronchoscope	Two dedicated bronchoscopes, equipment, and bronchoscope tower (labeled restricted use); IPaC staff–observed cleaning to ensure proper decontamination/terminal disinfection. Cleaning/decontamination of endoscope performed after each procedure: endoscope soaked in enzymatic detergent to remove soils (to reduce risk of splashing, no scrubbing performed); decontaminated endoscope placed in AER with a biologic indicator testing (to ensure proper decontamination/cleaning); each endoscope load underwent 2 cycles in the AER before reuse.
Medical waste	All medical waste placed in RMW bags located inside patient's room, sprayed, and then placed in a rigid plastic container (labeled biohazard/RMW) before disposal and incineration, following Germany's regulations for handling infectious biohazardous wastes. Disposable sharps placed in sharps containers, autoclaved, and contained in protected location until disposal/decontamination (incineration). Suctioned containers holding blood-contaminated fluids, oral and respiratory secretions, bronchoscopy drainage fluids, or other drainage from patient snapped closed and contained/stored/labeled as biohazard/RMW before disposal/incineration.
Linen	All linen (disposable isolation gowns of HCWs, sheets and gowns of patient) placed in labeled regulated medical waste bags and sealed. When full, these RMW bags were then stored in larger (50 gallon) RMW containers and secured in a RMW holding area (another AIIR in the ICU that was labeled and secured as a RMW holding area) until transport for incineration. The outside of all RMW bags/containers wiped down with a 10% bleach solution before transport.
Medical laboratory	Phlebotomy/laboratory tests limited to most critical samples; performed by a single laboratory technician. All specimens placed in a plastic zip-locking bag that was placed inside a rigid plastic container and then inside a second similar (but larger) plastic container with lid taped to the container (biohazard/RMW labels). All specimens directly transported to the laboratory. Except for blood and urine samples for diagnostic tests, specimens not pretreated with polyethylene glycol p-tert-octylphenyl ether under a laminar flow hood to reduce viral load before shipment to Bernard Nocht Institute because of concern treatment may interfere with validity of laboratory tests (but will be recommended in future cases). PPE for laboratory workers included gown, gloves, and N95 respirators (N95 respirators worn because specimens with a high viral load were tested in analyzers outside the BSC). Centrifugation of specimens performed within a Class II BSC. Chemical disinfection of instruments/equipment performed immediately after each use with 10% bleach solution (or per manufacturer's recommendation). All specimens and nonreusable equipment autoclaved before disposal, then incinerated per Germany's regulatory requirements.
Terminal decontamination	Bleach 10% solution or standard hospital-grade disinfectants used for terminal cleaning of all surfaces and equipment, of patient's room, and of aero-evacuation airplane. Terminal cleaning of ICU room overseen by IPaC staff.
Cadaver	Body sprayed with 10% bleach, placed in a body bag that was then decontaminated with 10% bleach solution, and then in a second sealed body bag that was decontaminated with 10% bleach solution before transfer to morgue. Embalming performed (generally not recommended due to exposure risk) by personnel wearing biohazard suits with hood, full face respirators, and double gloves overlapping sleeves of biohazard suit (duct-taped at wrists). Embalming procedures observed by IPaC staff. Body maintained in room at 1.1°–3.3°C. Daily RT-PCR of serum samples and RT-PCR of deep tissue samples on days 1 and 6 after embalming (confirmed negative). Chemical disinfection of nonsurgical instruments and equipment; surgical instruments also sterilized. Terminal decontamination of room.

*AER, automatic endoscope reprocessor; AIIR, airborne-infection isolation room; BSC, Class II biosafety cabinet; HCWs, health care workers; ICU, intensive care unit; IPaC, infection prevention and control; PAPRs, powered air-purifying respirators; PPE, personal protective equipment; RMW, regulated medical waste; RT-PCR, reverse transcription PCR.

and chest tube placement. Infection prevention and control staff provided refresher training on the proper donning and doffing of personal protective equipment (PPE) and oversaw the decontamination of bronchoscopes and ventilators. The procedures for disposing of medical wastes were in accordance with German regulations for handling infectious biohazardous materials (Table 2).

Laboratory interventions involved limiting blood draws and analyses to the most critical samples and to a single laboratory technician. Laboratory personnel wore gowns, gloves, and N95 respirators. Centrifugation of specimens was performed within a Class II biosafety cabinet. Laboratory equipment was decontaminated immediately after use and all nonreusable equipment was autoclaved

before disposal. Blood and urine samples were pretreated with polyethylene glycol (to reduce viral load) before being shipped to Bernard Nocht Institute (Hamburg, Germany) for CCHF diagnostic testing. The cadaver was placed in 2 sealed body bags; the outside of each bag was decontaminated with a 10% bleach solution. RT-PCR analysis of deep cadaver tissue samples was performed after embalming and confirmed to be negative. Bleach (10%) or standard hospital-grade disinfectants were used for terminal cleaning of the patient's room and all surfaces and equipment in the airplane used to aero-evacuate the patient to Germany.

Outbreak Investigation

Contact tracing commenced immediately after diagnosis and included a wide group of persons who may have been at risk for exposure to the patient's blood/body fluids: personnel in the patient's deployed unit, persons at the Combat Support Hospital in Kandahar, the medical evacuation team, and persons at LRMC (HCPs, laboratory workers, and transport, housekeeping, and volunteer staff). Among these contacts, 18 HCPs were identified as having been at risk for exposure and were offered oral ribavirin PEP (off-label use); 16 of the 18 accepted treatment (Table 3). Most of the 18 HCPs were present during bronchoscopies or ventilation procedures that used a bag-valve-mask device and had reported blood splashes on their gowns. Although there were no known percutaneous exposures, 2 HCPs reported blood on intact skin. Also, some HCPs wore only a surgical mask as PPE during the initial bronchoscopies and/or were unsure if they had always maintained a properly fitted N95 respirator during subsequent bronchoscopies. The group of 16 HCPs who accepted ribavirin PEP included a medic

in Kandahar who had a blood exposure on his ungloved hand during an emergency intravenous catheter insertion and a physician in Kandahar who emergently intubated the patient without wearing an N95 respirator. The group also included an LRMC ICU nurse and respiratory therapist, both of whom had met the patient on arrival at LRMC and manually ventilated him during transport to the ICU without wearing a mask or eye protection; during the transport, the patient was actively bleeding from intravenous catheter sites and coughing blood into the endotracheal tube. These 2 HCPs (and others) were also present during the initial 2 bronchoscopies, during which they may not have worn surgical masks at all times (and no eye protection) and their gowns had been soaked from blood exposures. In addition, the respiratory therapist's face shield dislodged immediately after being sprayed with blood while she was manually ventilating the patient using a bag-valve-mask device during a life-threatening hypoxic event. The ICU nurse also had blood contact on her skin (wrist) during resuscitation, when her gown sleeve slipped from the glove. The respiratory therapist and ICU nurse were also among the HCPs, aside from a few physicians, who spent the most time directly caring for the patient. The remaining 72 personnel had unlikely/no identifiable exposure risk and were instructed to have their temperatures taken twice daily for 15 d and to contact the infectious diseases physician for any febrile illness within this same time period (Table 3).

An oral ribavirin PEP regimen of 600 mg twice daily for 7 d was recommended initially; this dosage was based on drug availability, drug tolerance, and dosage regimens reported in the literature (3). Seventy-two hours later, a more oral ribavirin became available, and the 16 HCPs

Table 3. Surveillance criteria and PEP, by exposure risk, for contacts of US soldier with fatal Crimean–Congo hemorrhagic fever, Germany, 2009*

Group no.	No persons	Risk	PEP and monitoring
1	18	Contact of skin or mucous membranes with contaminated blood or body fluids; present during bronchoscopy or during use of bag-valve-mask ventilation device (risk of aerosolization of infectious blood/body fluids likely) and without proper PPE†	Oral ribavirin PEP offered; baseline and at least weekly chemistries and CBC; CCHF acute/convalescent-phase titers‡; monitoring for fever (twice daily) and for CCHF symptoms and medication side effects (for 15 d in clinic)
2	31	Present during bronchoscopy or during use of bag-valve-mask ventilation device (even with proper PPE)†; known contact with contaminated blood or body fluids but wore proper PPE and without PPE breaches† (no known mucosal or skin contact with infectious blood/body fluids); laboratory workers who performed tests on specimens (removed specimens from container) and wore proper PPE†	Monitoring for fever twice daily for 15 d (in clinic); self-observation and reporting of signs or symptoms e.g., fever) for 15 d
3	41	Persons in patient's room who wore proper PPE and without PPE breaches and no contact with infectious blood/body fluids†; laboratory workers who handled laboratory specimens (but did not remove specimens from container) and wore proper PPE†	No active monitoring; self-observation and reporting of signs or symptoms (e.g., fever) for 15 d

*CBC, complete blood count; CCHF, Crimean–Congo hemorrhagic fever; PEP, postexposure prophylaxis; PPE, personal protective equipment.

†Proper PPE for aerosol exposure included gown, gloves, N95 respirator, and protective eyewear; powered air-purifying respirators and full biohazard suits were required during bronchoscopies and chest tube placements by physician performing the procedure.

‡ELISA for CCHF-specific IgM and IgG performed at the Centers for Disease Control and Prevention, Atlanta, Georgia, USA (11).

were offered a 4-times-daily dosing regimen (600 mg/dose) and/or extension of PEP from 7 to 14 d. Because of the drug's side effects, all HCPs chose to remain on a twice-daily dosing regimen; only 2 HCPs accepted an extension of PEP to 14 d. Side effects (mainly fatigue, dyspepsia, nausea, and headache) were reported by all 16 HCPs. Of the 12 HCPs compliant with blood draws, 10 showed an increase in total bilirubin (range 1.2–5.7 mg/dL) and 2 had mild anemia (nadir hemoglobin 11.9 g) attributed to hemolysis caused by ribavirin. Leukopenia was observed in 1 HCP (leukocyte count 2,800 cells/mm³).

To assess possible seroconversion in the patient's contacts, initial and follow-up (4–6 wk) blood samples were obtained from personnel in the patient's deployment unit (n = 62), persons at the Combat Support Hospital in Kandahar (n = 55), and persons at LPMC (n = 74) and sent for serologic testing at the Centers for Disease Control and Prevention (Atlanta, GA, USA) (11). Although baseline serologic testing was not done, results of serologic testing done at 8 weeks for 2 HCPs who received oral ribavirin PEP (the ICU nurse and the respiratory therapist at LPMC) were consistent with acute CCHF seroconversion: CCHF virus-specific IgM and IgG titers were $\geq 6,400$, and over the next 2 mo, IgM titers declined (Table 3). These 2 HCPs were the most symptomatic of the 16 persons who received ribavirin PEP, and the only persons to seek medical attention for their symptoms. Symptoms were initially noted 4–5 d after exposure (day 4 of ribavirin PEP). The ICU nurse had moderate abdominal discomfort and jaundice (total bilirubin of 5.7 mg/dL; direct bilirubin 0.2 mg/dL; reference values for alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, leukocytes, and platelets). The respiratory therapist experienced fatigue, myalgias, and chills (no documented fever). She had a reference bilirubin level and mild leukopenia (leukocytes decreased from 4,200 cells/mm³ to 2,800 cells/mm³), and she missed 3 d of work because of her symptoms. For both HCPs, the symptoms and laboratory abnormalities were initially attributed to side effects of ribavirin therapy (particularly the elevated bilirubin level and gastrointestinal discomfort); in retrospect, the respiratory therapist's symptoms most likely represented CCHF symptoms ameliorated by ribavirin PEP. Ameliorated CCHF as a cause of symptoms in the ICU nurse could not be excluded.

Discussion

This fatal case of CCHF in a US soldier illustrates several issues regarding clinical management, infection control measures, epidemiologic investigation, and ribavirin PEP in CCHF infection. Early recognition and diagnosis of CCHF is paramount, so that medical care and appropriate infection control measures can be implemented in the initial phase of illness and, thereby, improve survival and

prevent nosocomial transmission. Delayed diagnosis and implementation of infection control measures can result in the need for extensive public health resources to evaluate and follow up on exposed HCPs and contacts (12–17).

The greatest risk for nosocomial transmission of the CCHF virus has been from percutaneous exposure with contaminated needles. Blood exposure on intact skin is a much lower risk, but sporadic transmission has been reported after skin or mucosal exposures to infectious blood. Nosocomial infection has also been reported without a clear source of virus transmission, and possible droplet (patient-to-patient) transmission has been reported (3).

Bronchoscopies and other procedures producing infectious aerosols were potential sources of CCHF virus transmission to the 2 HCPs reported here; however, no other HCPs who were present during the initial 2 bronchoscopies showed seroconversion. Thus, it is probable that virus transmission resulted from exposure to infectious blood during initial transport of the patient to the ICU, when neither HCP wore proper PPE (surgical mask/N95 respirator) while manually ventilating the patient or from a breach in PPE (particularly, for 1 HCP when her face shield dislodged during a resuscitation procedure). Of concern, both HCPs were unaware of their PPE breaches/exposures during resuscitation efforts; they were noted by other HCPs. The PPE breaches and potential blood/body fluid exposure risks led to the use of biohazard suits and powered air-purifying respirators during subsequent bronchoscopies and chest-tube placements. Compared with face shields, powered air-purifying respirators are less likely to become dislodged. The 2005 Centers for Disease Control and Prevention's modified PPE guidelines for suspected VHF at US hospitals note that extenuating circumstances (i.e., procedures generating aerosols, severe pulmonary involvement, or copious bleeding) may necessitate an increase in PPE (i.e., plastic aprons, leg/shoe coverings) or airborne precautions (9).

The processing of serum specimens with high viral loads in analyzers outside the Class II biosafety cabinet in a non-negative-pressure laboratory was a concern. Pretreatment of specimens to reduce viral load (i.e., with heat inactivation or polyethylene glycol) will be recommended in future cases, even though such treatment may affect laboratory results (9,18). Also, the CCHF virus has been detected in urine by RT-PCR as late as day 36 after illness onset; however, infectivity has been unclear because the virus had not been previously cultured from urine (19,20). The culture of the CCHF virus in patient samples obtained on day 6 of illness indicates that a positive RT-PCR result for urine may represent viable virus and the potential for late transmission of virus by patients who have recovered from CCHF.

Ribavirin, a synthetic purine nucleoside analog, has demonstrated in vitro activity against CCHF virus and

decreased death rates in infected suckling mice (3). Ribavirin efficacy in humans has not been evaluated in placebo-controlled trials against CCHF because of ethical concerns. However, retrospective analyses of ribavirin-treated CCHF virus-infected cohorts (compared with untreated historical controls) often report a decrease in CCHF-associated death if given within 72 h after the onset of symptoms (3). Anecdotal reports of ribavirin PEP in CCHF cases are limited, but they also suggest a possible benefit in preventing or ameliorating disease in most HCPs (2,3). However, the optimal dosage and duration of oral ribavirin for CCHF prophylaxis is unknown. PEP regimens in the literature range from 200 mg twice daily to as high as 4 g daily for 5–14 d; the most common regimen is 500 mg 3–4 times/d for 7–10 d. A 2-g loading dose is recommended in some regimens, particularly when treatment initiation is delayed (3,21). Drug side effects (mainly gastrointestinal intolerance and fatigue) often limit the dosage and duration of ribavirin PEP (3).

CCHF virus IgM and IgG titers in the 2 seropositive HCPs corresponded to acute infection from nosocomial transmission because these persons had no other risk factors for recent exposure to the virus. The mild/absent CCHF symptoms in these 2 HCPs who received a lower dose and duration of ribavirin PEP (600 mg twice daily for 7 d) may provide further insight regarding the potential benefit and dosage regimen for oral ribavirin PEP. The estimation of 88% of CCHF cases in Turkey being subclinical in a recent seroprevalence study would likely necessitate a controlled clinical trial to assess the efficacy and dose for ribavirin PEP (22).

On arrival at LRMC (day 6 of illness), the patient had a poor prognosis for survival: ribavirin treatment had been delayed ≥ 4 d after symptom onset; he was somnolent; and he had severe bleeding and coagulopathy, significantly elevated levels of hepatic transaminases, a platelet count of $\leq 20,000/\text{mm}^3$, and a serum viral load of $\geq 1 \times 10^8$ copies/mL (4,23–28). However, with supportive care, the patient showed clinical improvement (i.e., improved respiratory status, decreased bleeding and blood product requirements, and improved end-organ function). Continuous renal replacement therapy was particularly helpful in controlling the patient's life-threatening metabolic derangements; hepatic replacement therapy was of uncertain benefit and interfered with the optimal use of continuous renal replacement therapy. On day 10 of illness, the patient was able to follow commands, his serum viral load was declining, and CCHF-specific IgG was present.

There were multiple possible reasons for the fatal brain herniation on day 11 of illness. CCHF infection can cause endothelial cell dysfunction (with increased vascular permeability) through the induction of cytokines (tumor necrosis factor- α , interleukin-6, interleukin-10, interferon

[IFN]- γ), which can result in cerebral edema (28,29). Increase in these cytokines and markers for increased endothelial cell permeability (i.e., vascular endothelial growth factor [VEGF]-A and soluble VEGF receptor 1) have been correlated with increased serum viral load and increased risk of death and/or severe CCHF disease (Table 2) (30–36). In a similar VHF case (Marburg virus disease) with elevated levels of cytokine and soluble VEGF receptor 1 in which brain herniation occurred, the patient had cerebral edema that was not controlled with renal and hepatic dialysis, mannitol, and hypotonic saline (37). Other contributing factors for cerebral edema include possible direct effects of the virus in the brain and the combination of hepatic failure, persistent acidosis, and large osmotic shifts caused by dialysis; frontal lobe hemorrhage was not a significant factor because of its small size and location.

Severe CCHF has been attributed to a delayed IFN- α response in infected persons and to insensitivity of infected cells to the effects of the response (i.e., down-regulation of the host's innate immune response) (38,39). The failure of ribavirin to prevent death in mice lacking type I IFN- α receptors (serum viral load was reduced, only delaying death), its poor ability to cross the blood–brain barrier, and its delayed initiation in this soldier, suggest a minimized effect of ribavirin against CCHF virus in this fatal case (3,40). However, a newer antiviral drug, favipiravir (a nucleoside analog also known as T-705), may offer promise as a future treatment option for CCHF. Mice lacking type I IFN- α receptors had no detectable CCHF-specific antibody if given favipiravir within 2 d of CCHF virus challenge, and all mice treated within 3 d of challenge survived with no detectable virus in the blood or organs (40).

This case of a soldier who died from CCHF illustrates the need to maintain an index of suspicion for CCHF and other VHFs in febrile travelers returning from VHF-endemic areas so that supportive care and appropriate infection control measures can be implemented early in the course of illness. This case highlights the critical care challenges in caring for a patient with severe CCHF and describes nosocomial CCHF virus infection in 2 HCPs who were receiving oral ribavirin PEP (600 mg twice daily). The 2 nosocomial infections stress the need for infection control policies that educate HCPs to use contact and droplet precautions (minimal requirements) when caring for patients presenting with fever and hemorrhage. In tertiary-care medical settings, procedures and emergency resuscitations performed on VHF patients with severe hemorrhage may pose risks for HCPs that are different from those in smaller hospitals in developing countries, where such procedures may not be available. Because the risk for aerosol production, splashing blood, and breaches in PPE (i.e., dislodged face shields, face masks, sleeve separation from gloves) is highest during resuscitative efforts, in this VHF case with

severe hemorrhage, Tyvek suits with powered air-purifying respirators were indicated for the HCPs at highest risk for possible exposure to infectious materials (i.e., bronchoscopes). Although in this case, the potential antiviral effect of ribavirin may have been decreased because of late initiation of the drug and poor penetration of the blood–brain barrier, ribavirin may have contributed (along with CCHF IgG) to the patient’s improved clinical condition and decreased serum viral load. In addition, the probable CCHF virus seroconversion of 2 HCPs who had ameliorated or no symptoms after receiving ribavirin PEP may contribute further to the experience with and dosing regimen for ribavirin PEP in HCPs exposed to CCHF virus.

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Epidemiology and Ecology of Tularemia in Sweden, 1984–2012

Amélie Desvars, Maria Furberg, Marika Hjertqvist, Linda Vidman, Anders Sjöstedt, Patrik Rydén, and Anders Johansson

The zoonotic disease tularemia is endemic in large areas of the Northern Hemisphere, but research is lacking on patterns of spatial distribution and connections with ecologic factors. To describe the spatial epidemiology of and identify ecologic risk factors for tularemia incidence in Sweden, we analyzed surveillance data collected over 29 years (1984–2012). A total of 4,830 cases were notified, of which 3,524 met all study inclusion criteria. From the first to the second half of the study period, mean incidence increased 10-fold, from 0.26/100,000 persons during 1984–1998 to 2.47/100,000 persons during 1999–2012 ($p < 0.001$). The incidence of tularemia was higher than expected in the boreal and alpine ecologic regions ($p < 0.001$), and incidence was positively correlated with the presence of lakes and rivers ($p < 0.001$). These results provide a comprehensive epidemiologic description of tularemia in Sweden and illustrate that incidence is higher in locations near lakes and rivers.

Tularemia is a zoonotic disease that causes geographically confined and seasonal outbreaks in many locations in the Northern Hemisphere (1–3). The highly infectious causative bacterial agent, *Francisella tularensis*, comprises 4 subspecies, but nearly all cases of tularemia are caused by subspecies *tularensis* (type A), the most virulent type, which is found in North America, or subspecies *holarctica* (type B), which is the most widespread species in Europe (4). *F. tularensis* can infect humans through bites of arthropods (e.g., mosquitoes, ticks, tabanid flies); inhalation of infectious aerosols; handling of infected animals; or ingestion of contaminated water (2,3).

Sweden, Finland, and Turkey have reported the highest incidences of tularemia worldwide (5). In Sweden and Finland, the most common form of the disease is ulceroglandular tularemia, which is characterized by a skin ulcer at the site of infection and adjacent swollen regional lymph

nodes (6–8). A marked seasonality of tularemia has been reported in Sweden; most cases occur during late summer and early autumn (8–12). An exception was an outbreak affecting >2,700 persons during late fall and winter in 1966–1967 (13). In 2000, large numbers of cases were recorded outside the historically tularemia-endemic northern regions of Sweden, which could indicate a changing geographic pattern of disease (6).

F. tularensis subspecies *holarctica* naturally infects several mammalian wildlife species, in particular, mice, rabbits, hares, beavers, voles, lemmings, and muskrats (14). In Europe, the ticks *Dermacentor reticularis* and *Ixodes ricinus* are vectors for the bacterium (15–17), although previous research has suggested that mosquito bites are the most frequent route of transmission to humans in Sweden (6,9,18). Furthermore, a relationship between exposure to *F. tularensis* and the presence of lakes and rivers has long been suspected and is repeatedly described in the literature on tularemia (4,19–23). However, the ecologic cycles and environmental reservoirs of tularemia remain largely unknown. Since the 1950s, observed disease patterns have suggested that tularemia foci in nature coincide with a suitable ecosystem at a particular place (21,24,25). According to this theory, disease vectors, hosts, and the pathogen are tied to a particular landscape—that is, an ecologic region—as the environmental determinant that controls disease distribution.

To determine the ecologic factors that contribute to the transmission of *F. tularensis* and the spread of tularemia in Sweden, we examined trends in the epidemiology of tularemia among humans during 1984–2012. We analyzed descriptive epidemiologic data, including the geographic distribution of cases during the study period, and investigated if changes in distribution occurred and if disease was associated with particular ecologic regions and inland water.

Methods

Sources of Epidemiologic, Geographic, and Demographic Data

Since 1968, suspected and confirmed tularemia cases have been mandatory reportable diseases as required by the

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Swedish Communicable Disease and Prevention Act. Data on tularemia cases occurring from September 1984 through December 2012 were collected from the national system for communicable disease surveillance database, maintained by the Public Health Agency of Sweden. Data on patient sex, age, place of residence, suspected location of disease exposure, date of exposure, date of onset of illness, date of diagnosis, and date of notification were retrieved from this database. This study was approved by the Regional Ethical Review Board in Umeå, Sweden (2014-204-31M).

Data on population, land, and water areas were retrieved from Statistics Sweden (26). The proportion of land area covered by inland water (defined as lakes and rivers >6 meters wide) was determined by municipality. The areas of the 4 largest lakes of Sweden (Vänern, Vättern, Mälaren, and Hjälmaren) and sea water areas were calculated separately by municipality. Incidences of tularemia, nationwide and by municipality, were calculated on the basis of the number of infections per 100,000 persons and year by using population census data for 1984–2012. The altitude (meters above sea level) at which each tularemia case-patient was exposed to *F. tularensis* and the centroids of the 9,875 postal code areas in Sweden were retrieved by using the 30 arc-second digital elevation model of Europe (27) and the extraction toolset of the Spatial Analyst toolbox in ArcGIS version 10.0 (ESRI, Gävle, Sweden). Geographic data were visualized by using ArcMap software in ArcGIS and R software version 2.9.1 (<http://www.r-project.org>).

Geographic Coding and Spatial and Temporal Data

For each tularemia case recorded, geographic coordinates (longitude/latitude) were determined for the location of disease exposure and the disease onset date. (For details, see online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-0916-Techapp1.pdf>.) These data underwent quality coding to enable subsequent analysis with a high level of spatial and temporal certainty.

Ecologic Regions and Definition of Northern and Southern Sweden

For our study, we used previously defined ecologic regions (e.g., areas defined by the distribution of flora, fauna, geomorphology, climate, and soils) (28). The southernmost part of Sweden is nemoral forest (broadleaf forest); north of this region is the boreo-nemoral forest region (mixed deciduous and coniferous forest). Most of the rest of the country is boreal forest (coniferous forests) and is divided into 3 subregions: southern, middle, and northern boreal forest. Alpine tundra is located in northwestern Sweden and is composed of the birch forest, the middle to low alpine forest (grass and shrub heaths, fens), and the high alpine forest (boulder fields). We defined southern Sweden as the region located south of the southern border of the boreal

forest region; the area above this boundary was defined as northern Sweden (29).

Local Outbreaks and Outbreak Length

A local outbreak was defined as >4 cases of tularemia during a 30-day period in a municipality. The criterion for a new outbreak was a lag phase of ≥ 4 months after the end of the preceding 30-day outbreak period. The mean duration of outbreaks was compared between the northern and southern parts of Sweden by measuring the interquartile ranges of the outbreaks (i.e., the periods during which 50% of cases occurred).

Statistical Analysis

Categorical data were analyzed by using the χ^2 test for goodness of fit. Differences between incidence proportions were analyzed by using the 2-tailed 2-proportion z-test with a 95% CI, and nonparametric bootstrapping was used to construct a 95% CI for the relative increase in risk. The Wilcoxon rank-sum test was used to compare differences between groups, and the Spearman rank correlation was used to study the dependencies between variables. The spatial distribution of tularemia cases was compared with a regularly distributed set of points determined by the underlying population of each municipality. A municipality was part of an ecologic region if its geographic centroid was within the borders of the ecologic region. All statistical analyses were conducted in R version 2.9.1.

Results

Epidemiologic Characteristics of Tularemia in Sweden

During 1984–2012, a total of 4,830 cases of tularemia were notified to the Public Health Agency of Sweden; of these, 4,792 patients were infected in Sweden. The annual mean incidence of tularemia on the basis of these 4,792 patients was 1.86 cases per 100,000 persons (range 0.00–5.62; Figure 1). A total of 2,791 (58.2%) case-patients were men; mean age was 47.6 ± 19.5 years (men, 47.8 ± 19.5 ; women, 47.2 ± 19.5 ; range 1–95 years). After applying quality criteria for disease onset date and location of disease exposure, 3,524 of the 4,792 cases were included for the subsequent analyses (the quality of descriptive metadata connected with cases is summarized in online Technical Appendix Table 1). All further results, including incidence estimates, were determined on the basis of these 3,524 cases for which high-quality data on disease onset date and location of infection were available.

During the study period, tularemia incidence was distributed widely among age groups, with the highest incidence among those 55–69 years of age (Figure 2). The mean annual incidence by age group showed a distinct bimodal pattern for both sexes; peaks in incidence for age groups 10–14 and

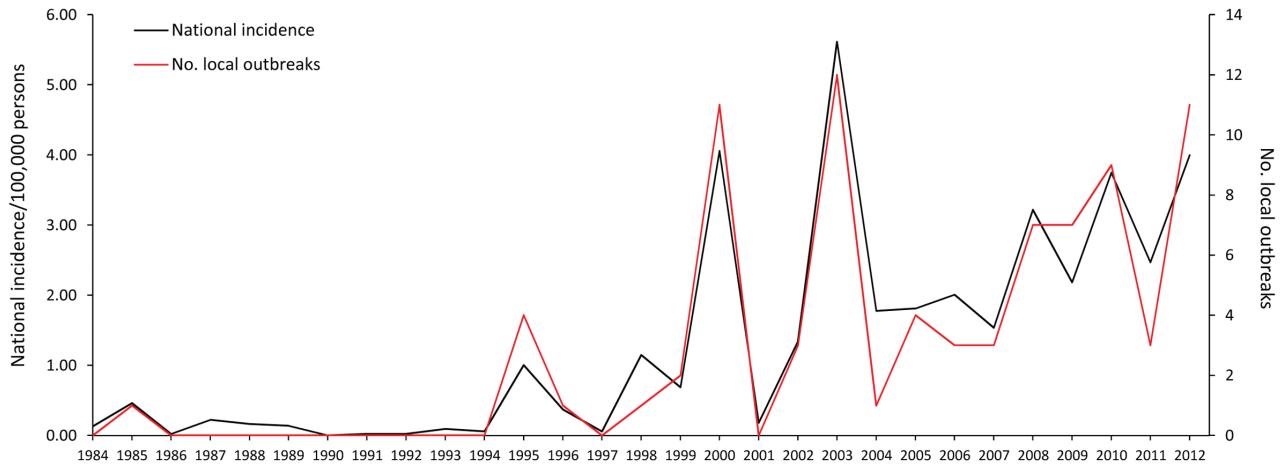


Figure 1. Mean incidence of tularemia (per 100,000 persons) and number of local outbreaks, Sweden, 1984–2012.

55–59 years were 0.93 and 2.75 cases per 100,000 persons, respectively. The global relative risk of contracting tularemia was 1.39 times higher for men/boys than for women/girls; the corresponding difference in incidence between sexes was 0.44 cases/100,000 persons/year (95% CI 0.35–0.53; $p < 0.001$). By age group, the incidence of tularemia for the study period was significantly higher for men than for women in all age groups ≥ 55 years of age (Figure 2). The male:female relative risk for infection was 0.89 for the 0- to 4-year age group but ranged from 1.15 to 4.28 for all other age groups.

Spatial and Temporal Distribution of Tularemia Cases

Tularemia reports were highly seasonal. The cumulative number of cases per week for 1984–2012 showed a

symmetric pattern with a peak at week 32; approximately two thirds of cases occurred during weeks 29–35 (mid-July to late August; Figure 3). The seasonal outbreak peaks were similarly distributed in the southern region (weeks 30 to 35) and in the northern region (weeks 30 to 34; Wilcoxon rank-sum tests, $p > 0.05$). The mean lengths of outbreaks were also similar between regions (Wilcoxon rank sum test, $p > 0.05$).

Tularemia was reported in 189 of 290 municipalities during the study period; some geographic clustering of cases was evident (Figure 4). Most cases were reported from the northern region, where $\approx 20\%$ of the Swedish population lives, and incidence was significantly higher in the northern region (4.52 cases/100,000 persons/year) than

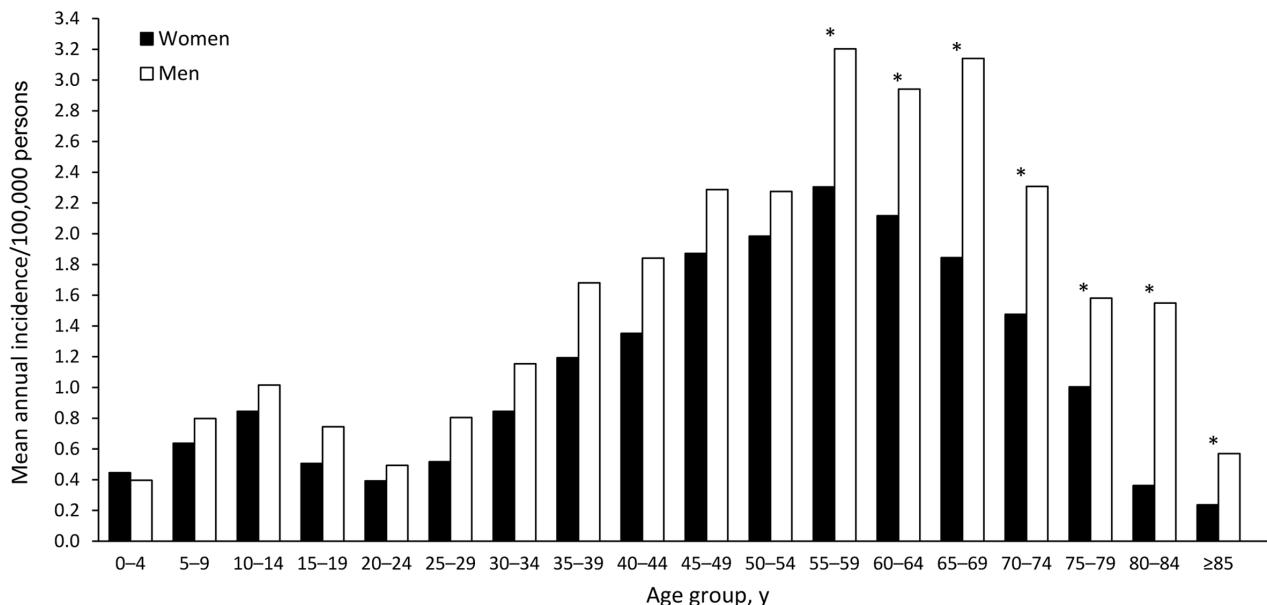


Figure 2. Annual mean incidence of tularemia by age group and sex, Sweden, 1984–2012. Asterisks (*) indicate significant differences by sex.

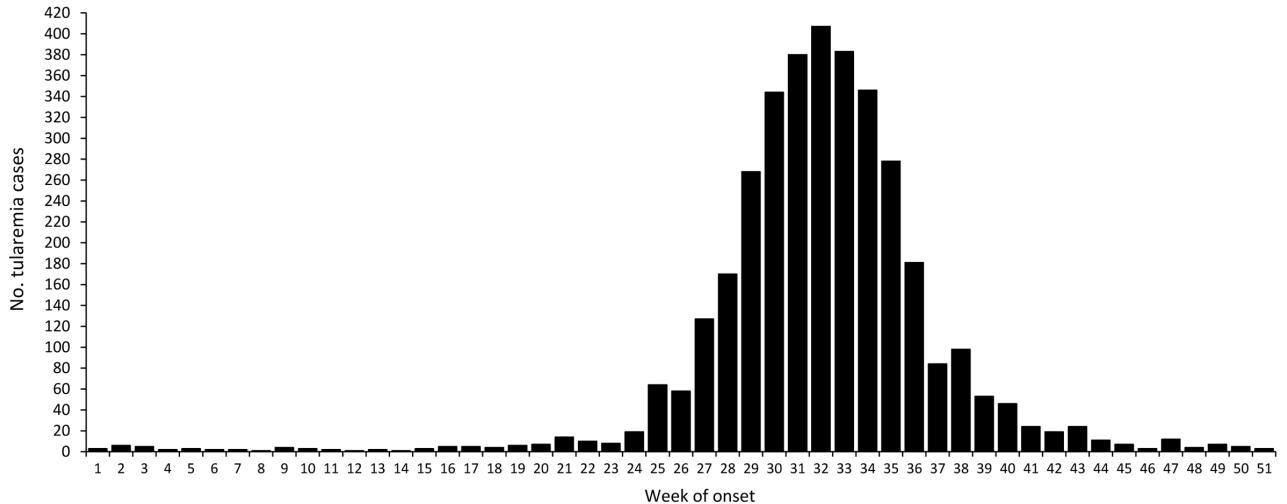


Figure 3. Cumulative number of tularemia cases, by week of onset, Sweden, 1984–2012.

in the southern region (0.56 cases/100,000 persons/year). The 95% CI for estimating the difference in incidence between the northern and southern regions was 3.77–4.14 cases/100,000 persons/year ($p < 0.001$). Denser case aggregates were found in northeast areas of Sweden and in a belt around the southern border of the boreal forest region, which includes the municipalities Ljusdal, Malung, Ockelbo, and Örebro (Figure 4).

The nationwide number of local outbreaks per year varied from 0 to 12 (mean 2.86 ± 3.80) and was largely correlated with the nationwide annual incidence, demonstrating that many local outbreaks occurred simultaneously during outbreak years (Figure 1). The number of outbreaks per municipality during 1984–2012 ranged from 0 to 9 (online Technical Appendix Table 2). The highest annual tularemia incidence per municipality was recorded in Ockelbo in 2000 (921 cases/100,000 persons), followed by Malung in 2003 (588/100,000 persons) and Ljusdal in 2008 (429 cases/100,000 persons) and 1998 (402 cases/100,000 persons) (Figure 4).

Analysis showed a significant long-term change in the annual mean incidence of tularemia from the first to the second half of the study period. Incidence was 0.26 cases/100,000 person/year during 1984–1998 but 2.47 cases/100,000 persons/year during 1999–2012 (Table). The 95% CI for estimating the difference in incidence between 1984–1998 and 1999–2012 was 2.16–2.36 cases/100,000 persons/year ($p < 0.001$). An analysis of incidence by municipality showed that, during the first half of the study period, tularemia was mainly reported from municipalities in northern Sweden (Figure 5). However, as the nationwide tularemia incidence increased in the second half of the study period, the disease occurred over a larger geographic area, extending into the southern region. The rate of increase in case reports during the study period was 9.6

times higher in the south than in the north (χ^2 test, 95% CI 6.37–16.93; $p < 0.001$).

Tularemia and Ecologic Regions, Altitude, and Inland Water

The incidence of tularemia was unevenly distributed among the 6 ecologic regions of Sweden (Figure 6). An even disease distribution based on 3,524 cases within the country corresponded with a mean incidence of 1.35 cases/100,000 persons/year. The observed incidences in the nemoral, boreo-nemoral, and combined boreal and alpine regions were 0.02, 0.80, and 4.61 cases/100,000 persons/year, respectively. The 95% CI for measuring the deviation from the mean incidence was -1.42 to -1.23 cases/100,000 persons/year ($p < 0.001$) for the nemoral region; -0.62 to -0.47 cases/100,000 persons/year ($p < 0.001$) for the boreo-nemoral region; and 3.05–3.47 cases/100,000 persons/year ($p < 0.001$) for the combined boreal and alpine region.

Exposure to *F. tularensis* occurred at a median altitude of 59.0 meters (range 0–900 meters); the densest case aggregates were distributed over different altitudes (Figure 7). For municipalities that reported tularemia cases, the mean incidence was 9.27 cases/100,000 persons/year, and a significant positive correlation was seen between the mean altitude of disease exposure and disease incidence (Spearman ρ 0.41, 95% CI 0.28–0.53; $p < 0.001$). The incidence was significantly higher than expected at altitudes > 100 meters and lower than expected at altitudes < 50 meters ($p < 0.001$). The 95% CIs for measuring the deviation from the mean incidence were 1.08–2.42 cases/100,000 persons/year at altitudes > 100 meters and -1.80 to -0.77 cases/100,000 persons/year at altitudes < 50 meters.

We found a positive correlation between the mean incidence of tularemia and the proportion of municipality area covered by inland water (Spearman ρ 0.36, 95% CI

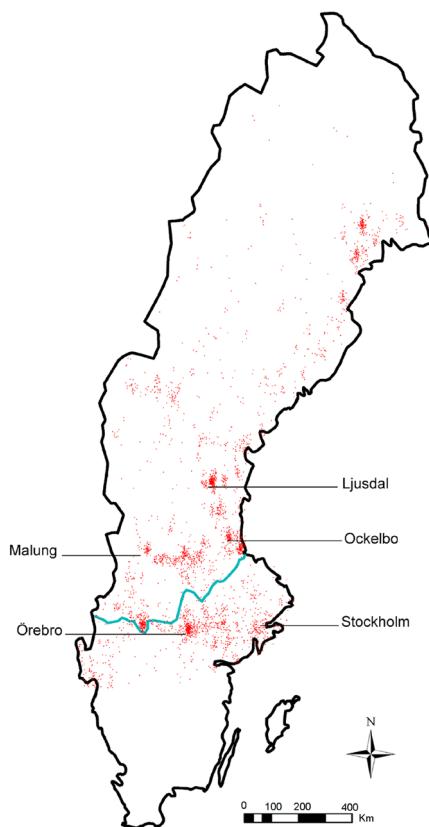


Figure 4. Distribution of 3,524 tularemia cases, Sweden, 1984–2012. Red dots indicate locations of reported cases; blue line indicates border between northern and southern Sweden, as defined by the southern border of the boreal forest. The municipalities with the highest tularemia incidence (Ljusdal, Malung, Ockelbo), and most outbreaks (Örebro) are indicated, as is the capital city of Stockholm.

0.23–0.47; $p < 0.001$) but a negative correlation between the mean incidence of tularemia and the proportion of the municipality areas covered by sea water (Spearman $\rho -0.28$, 95% CI 00.40 to -0.15 ; $p < 0.05$). We found no correlation between the mean incidence per municipality and the proportion of the municipality area covered by the 4 largest lakes in Sweden (Spearman $\rho -0.10$; $p > 0.05$).

Discussion

We used 29 years of nationwide notifiable disease surveillance data on tularemia in Sweden to investigate the epidemiologic

patterns in relation to time, disease location, and certain ecologic factors. The approach enabled us to identify a marked overrepresentation of cases in the northern part of the country, including annual incidences of 400–900 cases per 100,000 persons in some municipalities, and to identify a marked increase in the overall number of cases reported during the study period. We also observed that the rate of disease increase was higher in the southern part of the country than in the northern part, which suggests that tularemia is becoming more common in the southern regions. We also found statistical support for an association of tularemia with the location of lakes and rivers and with certain ecologic regions of Sweden, a finding notable because the existence of such associations has been postulated for decades but did not have robust support.

We determined that those 40–70 years of age were at greatest risk for tularemia, whereas, for unknown reasons, young adults were at the lowest risk and children and young teenagers were at intermediate risk. A similar age distribution was recently reported from Missouri, USA, which indicates that this type of age distribution is not unique to Sweden (30). Age-related differences in disease incidence could mirror differences in behavior; for example, higher-risk outdoor activities such as farming, hunting, and berry-picking may be more common among 40–70-year-olds.

The difference in incidence that we found between the sexes, with tularemia being more common in men than in women, is notable but not unique to this study. Similar findings were reported in several earlier studies (7,13,31). It is unclear if the sex bias results from a difference in exposure to disease or if men are simply more susceptible to tularemia. Our results demonstrate that differences by sex occurred across age groups for all case-patients ≥ 5 years of age, a finding that suggests that, among the possible biologic mechanisms involved, sex hormone differences are unlikely to explain the difference in incidence.

Results from our large set of case data corroborated previous reports on the seasonality of tularemia. The disease risk peaks in late summer or early autumn with most cases occurring in early August (6,7,9). In addition, we found that tularemia incidence was almost 10-fold higher in the second half than in the first half of the study period. We cannot rule out the possibility that improved disease awareness accounts for this difference, but because tularemia typically carries distinct symptoms, this is unlikely.

In agreement with previously published data, we observed that the tularemia incidence was highest in the

Table. Human tularemia notifications, by study period and geographic location, Sweden, 1984–2012*

Study period	No. cases (incidence)		All Sweden
	Northern Sweden	Southern Sweden	
First half, 1984–1998	324 (1.17)	19 (0.02)	343 (0.26)
Second half, 1999–2012	2,034 (8.61)	1,147 (1.14)	3,181 (2.47)
Full study, 1984–2012	2,358 (4.52)	1,166 (0.56)	3,524 (1.37)

*Only cases for which high-quality data on disease onset date and location of disease exposure were available were included. Incidence is given as no. case-patients/100,000 persons/year.

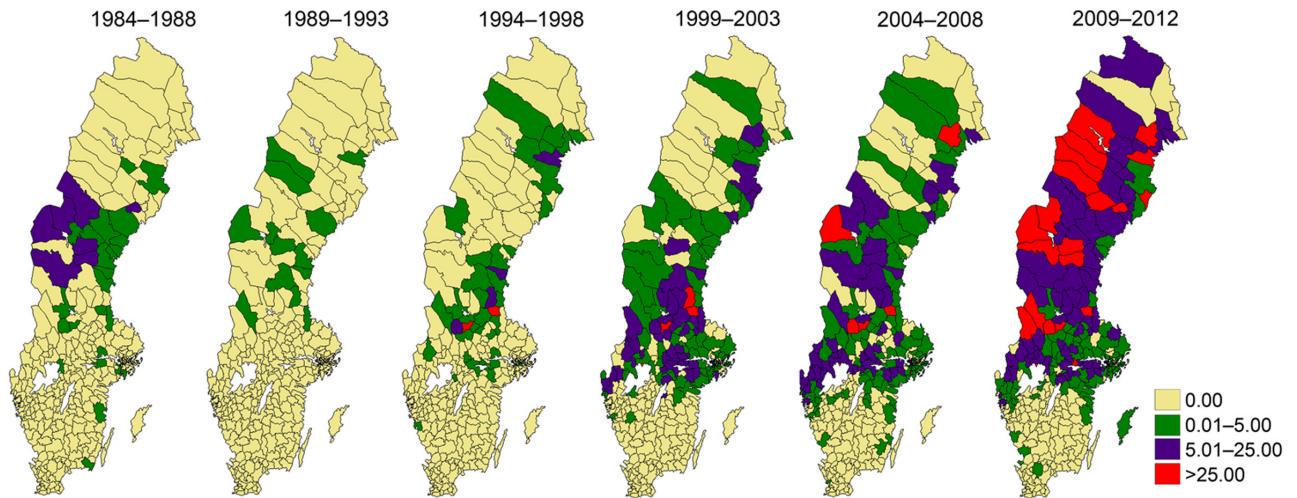


Figure 5. Mean incidence (per 100,000 persons) of tularemia in 189 municipalities by 5-year* intervals, Sweden, 1984–2012. *Most recent interval, 2009–2012, was 4 years.

northern part of Sweden and that the disease distribution was uneven, with some municipalities reporting multiple outbreaks (8–10,12). The high risk of contracting tularemia in some municipalities is noteworthy and indicates that tularemia is a public health issue in these locations. Twelve municipalities recorded maximum annual incidences of >100 cases per 100,000 persons and infection transmission occurred almost exclusively during a few summer weeks, findings that offer several possibilities for enhanced preventive measures. Future research efforts can lead to the development of outbreak prediction tools that can help public health authorities make timely decisions on campaigns informing the public on steps to take to prevent infection, such as avoiding exposure to mosquito bites (18).

During the second half of the study period, risk for tularemia increased 9.6 times more in southern areas than in northern areas, which indicates that the disease is becoming more common in the southern part of the country. From the available data, we cannot determine whether this shift is occurring because *F. tularensis* is dispersing to new areas or if more infections occurred in the south during the second period because ecologic conditions facilitated increased bacterial multiplication and spread (e.g., through infected arthropod vectors).

A link between tularemia caused by *F. tularensis* subspecies *holarctica* and the location of lakes and rivers was suggested by extensive field work investigating tularemia >50 years ago (19–22), but we could find little data to prove this association. We found that tularemia incidence at the municipality scale was positively correlated with the proportion of land area covered by inland water (lakes and rivers) but negatively correlated with the proportion of land area covered by sea water. The latter finding can be interpreted as a relatively low incidence of *F. tularensis* infection in municipalities

with a long coastline, a finding which contrasts with the distinct association of *F. philomiragia*, *F. novicida*, and some other *Francisella* spp. with sea water environments (4).

Interpretation of our finding of underrepresentation of *F. tularensis* exposure at altitudes <50 meters and overrepresentation at altitudes >100 meters is difficult. The most dense geographic case aggregates from 1984–2012 were observed over a range of altitudes, which indicates that intense disease transmission to humans appears to be only marginally restricted by altitude. To provide more informative data, future studies should target areas that experience repeated outbreaks of tularemia and aim for detailed analyses of local disease exposure altitudes and proximity to lakes and rivers. Compilation of chemical and physical characteristics of lake and river waters in areas where disease incidence is high might clarify what kinds of aquatic ecosystems are connected with *F. tularensis*.

We also examined a possible correlation of tularemia with ecologic regions, sometimes referred to as a landscape epidemiology of tularemia (21), and found that tularemia cases were overrepresented in the 3 boreal forest regions and the alpine region of Sweden. Combined with the findings described above, these data support a scenario in which the disease is closely related to certain (micro-) environments and ecologic systems (21,24,25).

The strengths of the study include the large sample size and 29-year study period; of the total number of 4,792 cases, 3,524 cases had high-quality descriptive metadata on date of disease onset and location of disease exposure. In 1967, Pollitzer provided a complete overview of the published literature on tularemia in the Soviet Union with greater total case counts, but the raw data used in these older studies are difficult to compare with modern infectious disease surveillance data (32). Limitations of our study include the

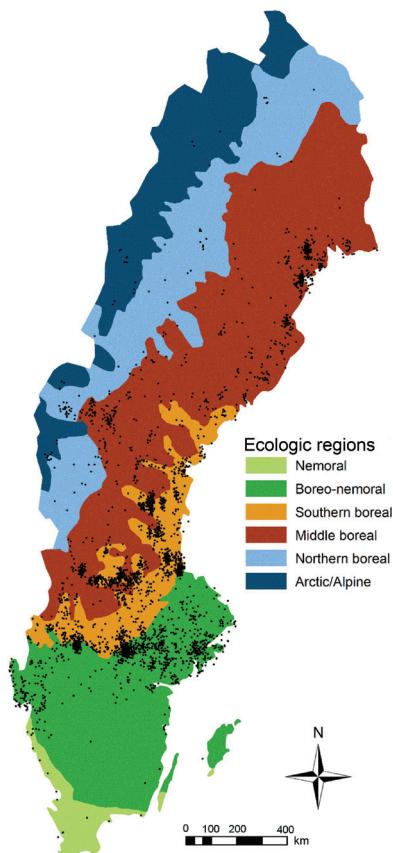


Figure 6. Distribution of tularemia cases by ecologic region, Sweden, 1984–2012. Black dots indicate locations of reported cases. Region designations adopted from (24).

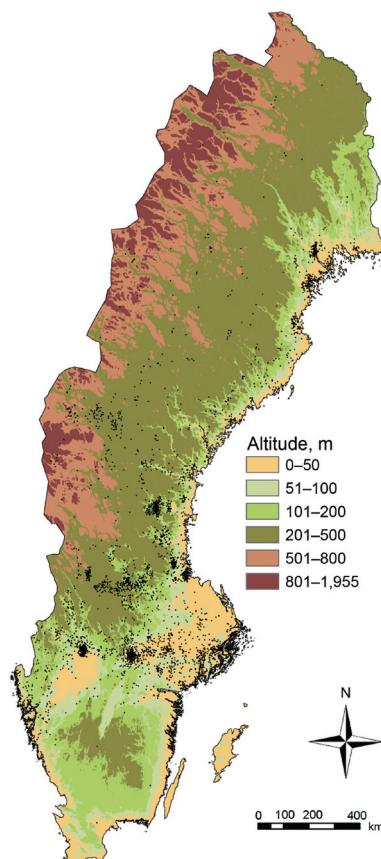


Figure 7. Distribution of tularemia cases by altitude, Sweden, 1984–2012. Black dots indicate locations of reported cases.

inherent weaknesses of infectious disease surveillance data in general; for example, data on risk factors related to human behavior are lacking, and disease in humans may be underreported because of few clinical symptoms (33,34). Some inaccurate or imprecise information on disease onset date and location of disease exposure may also have slipped through our filters to ensure strict data quality for case inclusion (e.g., because of patient recall bias).

In conclusion, our findings should stimulate discussion on future possibilities to prevent tularemia. Although this disease does not cause a high number of deaths, the illness can be incapacitating for days, weeks, and sometimes even months. Future studies should focus on the causes of an increased risk for disease in men and in older persons of both sexes. Our findings of a significantly increased risk for contracting tularemia in certain ecologic regions and the positive correlation between disease and inland water may prove useful in future prevention strategies. We believe that knowledge of ecologic region and proximity to water can be used to define areas within which tularemia exposure is more

likely. In addition, dynamics introduced by climate change, such as increasing temperature and changing precipitation patterns, can be incorporated in risk assessments (18,35). Finally, further study is needed to identify the reservoirs of *F. tularensis* in nature and the role of vector abundance. Human activities such as the restoration of wetlands and changes of land use may affect tularemia incidence, but data to influence appropriate risk assessments are lacking.

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Clinical, Environmental, and Serologic Surveillance Studies of Melioidosis in Gabon, 2012–2013

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Burkholderia pseudomallei, an environmental gram-negative bacillus, is the causative agent of melioidosis and a bio-threat agent. Reports of *B. pseudomallei* isolation from soil and animals in East and West Africa suggest that melioidosis might be more widely distributed than previously thought. Because it has been found in equatorial areas with tropical climates, we hypothesized that *B. pseudomallei* could exist in Gabon. During 2012–2013, we conducted a seroprevalence study in which we set up microbiology facilities at a large clinical referral center and prospectively screened all febrile patients by conducting blood cultures and testing for *B. pseudomallei* and related species; we also determined whether *B. pseudomallei* could be isolated from soil. We discovered a novel *B. pseudomallei* sequence type that caused lethal septic shock and identified *B. pseudomallei* and *B. thailandensis* in the environment. Our data suggest that melioidosis is emerging in Central Africa but is unrecognized because of the lack of diagnostic microbiology facilities.

The Tier 1 bio-threat agent *Burkholderia pseudomallei* is an environmental gram-negative bacillus and the cause of melioidosis, a disease characterized by sepsis, pneumonia, and abscess formation in almost any organ (1–3). *B. thailandensis* is closely related to *B. pseudomallei* but rarely causes disease in humans or animals; it is usually distinguished from *B. pseudomallei* by its ability to assimilate

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arabinose (4–6). Melioidosis mainly affects those who are in regular contact with soil and water and is associated with a mortality rate of up to 40% in resource-poor environments. The major regions to which melioidosis is endemic are Southeast Asia and tropical Australia (1,2). The northern tip of the Northern Territory in Australia and northeast Thailand represent hot spots, where annual incidence is up to 50 cases per 100,000 persons (1,7).

The emergence of melioidosis in Brazil is an example of increasing recognition of the disease in areas where it is probably endemic, and cases have become apparent as a result of enhanced awareness and diagnostics (1,8). Human *B. pseudomallei* infection has been reported from Malawi, Nigeria, The Gambia, Kenya, and Uganda; however, human cases in Africa seem to be few and isolated, although this finding could be the result of underrecognition and underreporting (1,9–12). Although reports of *B. pseudomallei* isolation from soil and animals in East and West Africa are limited, they suggest that melioidosis could be widely distributed across this region (13,14).

Given the equatorial tropical distribution of *B. pseudomallei* and *B. thailandensis*, we hypothesized that these bacteria are present in the central African country of Gabon, potentially causing disease. By conducting a seroprevalence study, an environmental survey, and setting up microbiology facilities for *B. pseudomallei* detection at a large referral hospital, we detected *B. pseudomallei* in soil and identified it as a cause of lethal infection in Gabon. We also detected *B. thailandensis* in environmental soil samples, indicating that this organism is also present in Gabon.

Methods

Study Sites and Populations

The study was performed in Moyen-Ogooué and Ngounié Provinces (combined population 162,000) in central

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Gabon; these 2 provinces cover an area of 56,285 km² and consist of predominantly dense primary rain forest. For the seroprevalence surveillance study, 304 serum samples were collected from healthy nonfebrile school children (12–20 years of age) living in and around Lambaréné, the capital of Moyen-Ogooué Province; these children also participated in a chemoprophylaxis study for malaria (15).

A prospective analysis of community-acquired bloodstream infections was performed at Albert Schweitzer Hospital (which admits ≈6,000 patients annually) in Lambaréné (population 24,000), in the Central African rain forest on the river Ogooué, Gabon. The rainy season starts in October and ends in June (including a short dry season in December–January). Mean annual rainfall is 1,981 mm (78 inches), which is equivalent to that in northeastern Thailand (16). Studies were approved by the Centre National de la Recherche Scientifique et Technologique, Libreville, and the scientific review committee of the Centre de Recherches Médicales de Lambaréné, Albert Schweitzer Hospital.

Prospective Analysis of Community-Acquired Bloodstream Infections

To obtain data about the prevalence and causes of community-acquired bloodstream infections in Lambaréné, we prospectively monitored all blood cultures for febrile patients admitted to Albert Schweitzer Hospital for 1 year (June 1, 2012–May 31, 2013) by using BacT/Alert PF (bioMérieux, Marcy l’Etoile, France). Criteria for ordering blood cultures were left to the discretion of the treating physician. Technicians and staff of the clinical microbiology laboratory received additional training on sample handling and processing (17,18). All oxidase-positive, gram-negative bacteria that were not *Pseudomonas aeruginosa* were further tested to determine whether they were *B. pseudomallei* by using the subculture and identification methods described below. Antimicrobial drug susceptibilities were determined by using Etest (bioMérieux) on Mueller-Hinton-agar (bioMérieux); when available, break points were defined as described (19).

B. pseudomallei Antibody Detection by Indirect Hemagglutination Assay

During May 2012, presence and titer of antibodies to *B. pseudomallei* in healthy schoolchildren were determined using by the indirect hemagglutination assay (IHA) as described (20,21), with pooled antigens prepared from 2 *B. pseudomallei* isolates from Thailand. An antibody titer of ≥1:40 was used as the cutoff value for seropositivity (22).

Soil Sampling Study

During July 2012–September 2012, soil sampling to test for the presence of *B. pseudomallei* was based on consensus

guidelines, and direct culture of soil in enrichment broth was performed (17,23). A total of 8 sites around the residences of children were selected on the basis of local maps and consultations with inhabitants throughout the provinces of Moyen-Ogooué (6 sites) and Ngounié (2 sites) and on known factors associated with the presence of *B. pseudomallei* (e.g., wet soil such as rice paddies or land use such as goat farming) (17) (Figure 1). Within each sampling area (50 × 50 m²), a fixed-interval sampling grid was used to collect 100 samples per field, 5 m apart. For each sample, 10 g of soil was collected from a depth of 30 cm, stored away from direct sunlight, and processed within 3 h.

Isolation of potential *Burkholderia* spp. from soil was performed as described (17,23). In brief, 10 g of soil was diluted in 10 mL of threonine–basal salt solution plus colistin at 50 mg/liter (TBSS-C50 broth) containing crystal violet and was vortexed for 30 s before incubation at

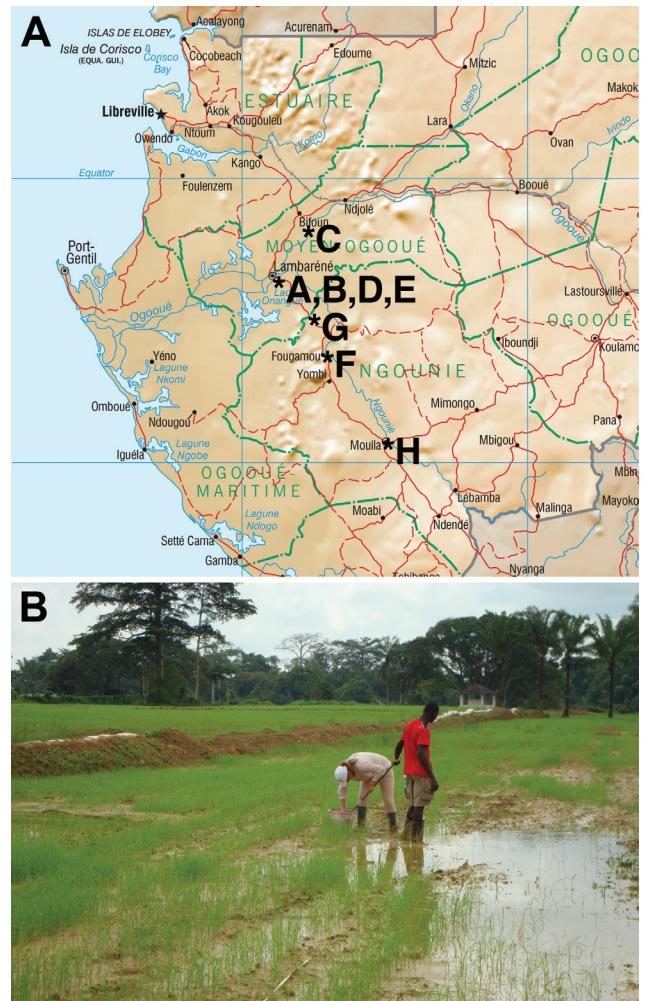


Figure 1. Environmental survey. A) Gabon, showing location of the 8 sites from which soil was sampled to test for the presence of *B. pseudomallei*, July 2012–September 2012. B) Soil sampling site no. H, a rice field near Mouila village.

≈42°C for 48 h. Ten mL of supernatant was subcultured onto Ashdown-agar and incubated and examined every 24 h for 7 days. *B. pseudomallei* was identified by colony morphology, positive oxidase test result, inability to assimilate arabinose, antimicrobial drug susceptibility pattern (*B. pseudomallei* is generally resistant to gentamicin and colistin but susceptible to amoxicillin/clavulanic acid [1,2]), and results of API 20NE (bioMérieux) and *B. pseudomallei*-specific (Bps) latex-agglutination tests (18,24,25). Positive results were confirmed with molecular analysis. Soil type was determined by standard lithologic and pedologic analysis of sediments; for this purpose, 2 extra samples were collected per site from a depth of 30 cm (26). Sediment properties were compared with properties of other (typical) samples from the same locations as described in the recently published Soil Atlas of Africa (26).

Genetic and Phylogenetic Analyses

Genomic DNA was extracted by using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) to perform multilocus sequence typing (MLST) (27). Primers used to amplify fragments of the 7 housekeeping genes were identical to those described at the *Burkholderia* MLST website (<http://bpseudomallei.mlst.net/misc/info2.asp>). For isolate *B. thailandensis* D50, the primer narK-up was replaced by narK-upAMC 5'-TCTCTACTCGTGCGCTGGGG-3'. Sequences of the 7 gene fragments of isolates from Africa were concatenated and combined with those from a selection of 971 sequence types (STs) representing all *B. pseudomallei*, *B. mallei*, and *B. thailandensis* isolates in the *B. pseudomallei* MLST database. Concatenated sequences were aligned and analyzed by using MEGA-6 (<http://www.megasoftware.net>). A phylogenetic tree was constructed by using a neighbor-joining algorithm and the Kimura 2-parameter model. Bootstrap testing was performed for 500 repetitions. Whole-genome sequencing was performed by using the MiSeq platform (Illumina, San Diego, CA, USA) as described (9).

Results

Community-Acquired Bloodstream Infections

Of the 941 bacterial blood cultures, 77 (8.2%) were positive for bacteria. The most prevalent isolate was *Escherichia coli*, responsible for 8 (10.0%) bloodstream infections, followed by *Staphylococcus aureus* (6 [7.8%]) and *Salmonella enterica* (6 [7.8%]), 5 of which were nontyphoidal salmonellae. Other organisms that were isolated at least 5 times included *Streptococcus pneumoniae* (5 [6.5%]), *Klebsiella pneumoniae* (5 [6.5%]), and *Enterobacter* spp. (5 [6.5%]). *B. pseudomallei* was isolated from 1 (1.4%) patient, described in the case report.

Case Report

A 62-year-old Gabonese woman was hospitalized in January 2013 with a 7-day history of fever, cough, weakness, headache, vomiting, and a painful knee. She did not report coughing or shortness of breath. She had poorly controlled diabetes mellitus and was taking glibenclamide. She had no history of cardiopulmonary or renal disease, was receiving no long-term medications other than glibenclamide, and did not smoke. She was a retired school teacher but still engaged in family farming. Physical examination revealed blood pressure of 160/90 mm Hg, a pulse rate of 130 beats per minute, and a temperature of 40.5°C. She had a wound with an underlying abscess on her right leg, together with diffuse tenderness of the right knee with warmth, erythema, and limitation of active and passive ranges of motion because of pain and effusion. Neurologic, cardiovascular, and respiratory examinations revealed no abnormalities. Laboratory findings obtained at admission showed an elevated blood glucose level of 24 mmol/L but values within reference range for creatinine (0.85 mg/dL), leukocytes ($9,800 \times 10^3/\text{mm}^3$), and hemoglobin (9.2 g/dL). No other blood or urine test was performed, and chest radiographs were not taken. On hospitalization day 1, treatment with amoxicillin/clavulanic acid was empirically initiated for sepsis. On day 2, the abscess was incised and drained, and on day 3 antimicrobial drug therapy was switched to ceftriaxone. Cultures of blood, wound, and synovial fluid grew identical gram-negative rods, which were initially classified as *Pseudomonas* spp. No other pathogens were detected. The patient's clinical condition deteriorated, and she died of septic shock on day 8. A postmortem examination was not performed.

After the patient's death, the *Pseudomonas* species was classified as *B. pseudomallei* (patient strain Gb100) and confirmed by MLST and whole-genome sequencing. This isolate was later determined to be susceptible to trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ceftazidime, and meropenem (Table 1).

Seroprevalence

Of the 304 healthy schoolchildren for whom serum samples were tested for *B. pseudomallei* antibodies, 143 (47.0%) were male. Details for this cohort have been reported previously (15). For 43 (14.1%) children, an IHA titer was detectable; titers ranged from 1:10 to 1:80 (median 1:10, interquartile range 1:10–1:20). For 5 (1.6%) children, IHA titer was $\geq 1:40$, which has been used as the cutoff value for seropositivity (22). None of the children had an IHA titer $> 1:160$, which is considered by several centers in Thailand to support a diagnosis of melioidosis in patients with clinical features consistent with this diagnosis.

Table 1. Antimicrobial drug susceptibility of *Burkholderia pseudomallei* and *B. thailandensis* strains from Gabon, 2012–2013*

Drug	MIC, mg/L			
	Break point resistance	<i>B. pseudomallei</i> patient strain	<i>B. pseudomallei</i> soil strain C2	<i>B. thailandensis</i> soil strain D50
Amikacin	4†	96	96	128
Tobramycin	4†	16	24	24
Ciprofloxacin	1	0.75	1.0	0.5
Moxifloxacin	1‡	0.75	0.75	0.75
Meropenem	4	0.75	0.75	0.75
Ceftazidime	8	2	2	2
TMP/SMX	1/19	1	1	1
AMC	8/2	4	4	6
TZP	32/?§	1.5	1.5	3
Chloramphenicol	8	3	3	3
Tetracycline	4¶	1.5	2	8
Polymyxin B	NA#	>1,024	>1,024	>1,024

*Bacterial isolates were tested for their susceptibility to antimicrobial agents. MIC (MICs; mg/L) were determined by E-test on Mueller-Hinton-agar. When available break points were defined as described [19]. AMC, amoxicillin/clavulanic acid; NA, not applicable; TMP/SMX, trimethoprim/sulfamethoxazole, TZP, piperacillin/ tazobactam.

†Break point for gentamicin was used.

‡Break point for ciprofloxacin was used.

§Break point available for piperacillin only.

¶Break point for doxycycline was used.

#Intrinsic resistance.

Environmental Isolates

The predominant soil type in this area of Gabon was ferralsol, which is red and yellow weathered soil. The only exception was samples taken from a rice paddy near Mouila village, where the soil was gleysol (clay, a hydric soil saturated with groundwater long enough to develop a characteristic gleyic color pattern) (Table 2).

B. pseudomallei was isolated from 21 (3%) of 800 soil samples taken from 3 (38%) of the 8 sample sites; the maximum number of positive samples for 1 site was 14 (14%) (Table 2). The biochemical profiles of all isolates were in accordance with *B. pseudomallei* (API 20NE code 1156576). The antibiogram of *B. pseudomallei* soil strain C2 is shown in Table 1.

Table 2. Geographic features and distribution of *Burkholderia pseudomallei* strains at 8 sampling sites in Moyen-Ogooué and Ngounié Provinces, Gabon, 2012–2013*

Site	Nearest village	Elevation, m	Land use	Soil type	Soil description	Sample holes positive, %
A	Lambaréné, Albert Schweitzer Hospital; lat. S 00°40'40.5, long. E 010°13'49.7	34	Football (soccer) field	Ferralsol	Yellowish-brown, clay fluvial sediments, not strongly humic, some gravel, poorly sorted sediment, decalcified	14
B	Lambaréné, Adouma; lat. S 00°40'50.2, long. E 010°13'31.5	14	Riverbed that is dry most of the year	Ferralsol, clay, orange, dry	Brownish yellow, clay fluvial sediments, moderately humic, some gravel, strong indicators of human interference	0
C	Makouké; lat. S 00°28'30.8, long. E 010°24'34.7	20	Cattle ranch	Ferralsol, orange, little stones, hard, rocky, less hard, orange	Yellowish brown, clay fluvial sediments, not strongly humic, some gravel, poorly sorted sediment, decalcified	4
D	Lambaréné, Adiwa; lat. S 00°41'06.0, long. E 010°13'43.5	8	Next to school (with Bps IHA positivity)	Ferralsol	Brownish yellow, clay fluvial sediments, moderately humic, some gravel, strong indicators of human interference	3
E	Lambaréné, Petit Paris 3; lat. S 010°42'40.4, long. E 010°15'20.7	35	Cattle ranch	Savannah/ ferralsol	Yellowish gray, well-sorted clay, weakly humic	0
F	Fougamou; lat. S 01°18'40.3, long. E 010°37'14.4	88	Savannah, grassland	Savannah/ ferralsol	Yellowish gray, well-sorted clay, weakly humic	0
G	Massika II; lat. S 00°40'40.7, long. E 010°13'51.4	55	Football pitch	Ferralsol	Reddish brown, clay fluvial sediments, not strongly humic, sediment, decalcified	0
H	Mouila; lat. S 01°51'27.8, long. E 011°02'37.7	92	Rice paddy	Gleysol	Greyish yellow clay with ferric concretions, gleyic features, probably associated with rice cultivation	0

*lat., latitude; long., longitude.

The closely related *B. thailandensis* coexists with *B. pseudomallei* in the soil in Southeast Asia and Australia and is generally considered avirulent (5,28). We also identified *B. thailandensis* in the soil of Gabon (Figure 2). This strain, termed *B. thailandensis* soil strain D50, was positive by Bps latex agglutination. This *B. thailandensis* strain, API 20NE code 1157577, was

susceptible to trimethoprim/sulfamethoxazole, amoxicillin/clavulanic acid, ceftazidime, and meropenem (Table 1).

Genetics and Phylogeny of *Burkholderia* spp. Strains

The 3 isolates from Gabon contained previously described MLST alleles but belonged to novel STs. The patient isolate Gb100 (ST1127) and soil isolate C2 (ST1128) were

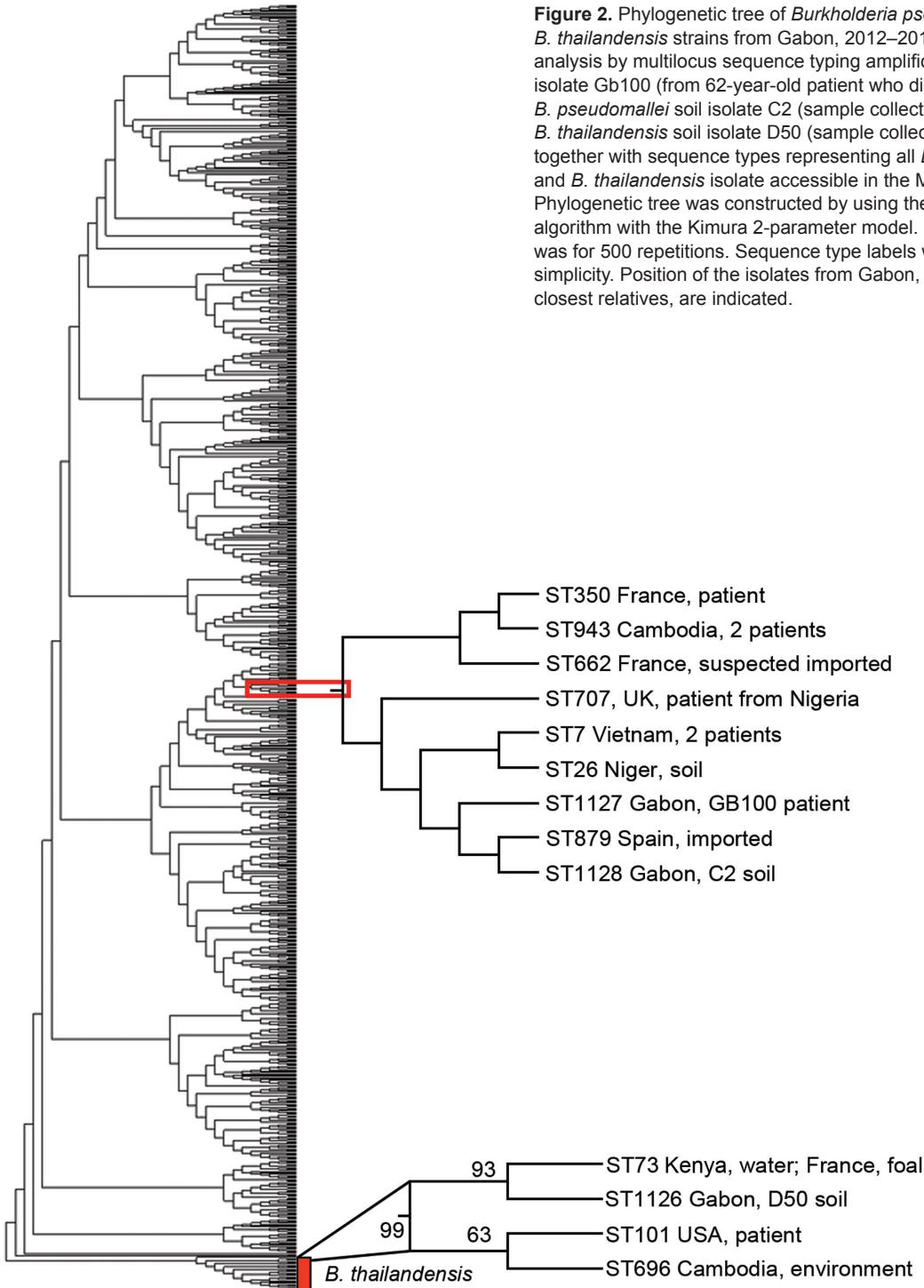


Figure 2. Phylogenetic tree of *Burkholderia pseudomallei* and *B. thailandensis* strains from Gabon, 2012–2013. Phylogenetic analysis by multilocus sequence typing amplification (MLST) of isolate Gb100 (from 62-year-old patient who died of melioidosis), *B. pseudomallei* soil isolate C2 (sample collected at site C), and *B. thailandensis* soil isolate D50 (sample collected at site D), together with sequence types representing all *B. pseudomallei* and *B. thailandensis* isolate accessible in the MLST database. Phylogenetic tree was constructed by using the neighbor-joining algorithm with the Kimura 2-parameter model. Bootstrap test was for 500 repetitions. Sequence type labels were omitted for simplicity. Position of the isolates from Gabon, including their closest relatives, are indicated.

single-locus variants and differed by 1 nt in the *narK* sequence only. Patient isolate Gb100 was also a single-locus variant of ST707 (single-nucleotide substitution in *ndh*). The only *B. pseudomallei* strain with ST707 in the database had been isolated in 2010 from a patient in the United Kingdom, 6 weeks after the patient had returned from a trip to Nigeria (12). The soil isolate C2 (ST1128) was a single-locus variant of ST7 (single-nucleotide substitution in *ndh*) and ST879 (single-nucleotide substitution in *lipA*). ST7 was represented by 2 isolates in the MSLT database, both isolated in 1963 from patients in Vietnam. The *B. pseudomallei* ST879 strain was isolated in 2011 from a patient in Spain, who had returned from a trip through Madagascar and 14 countries in West Africa (11). The soil isolate D50 (ST1126) was a single-locus variant of ST73. This ST is represented in the database by 2 *B. thailandensis* strains, 1 isolated from a foal in France and 1 isolated from the environment in Kenya. Phylogenetic analysis of the Gabon isolates together with 971 STs obtained from the MLST database by using the aligned concatenated sequences of the 7 loci in the neighbor-joining algorithm with the Kimura 2-parameter model showed that the patient isolate Gb100 and soil isolate C2, found near the community of the patient, grouped together with 7 STs. These 7 STs represented 10 *B. pseudomallei* strains isolated in Cambodia (2 strains), Vietnam (2 strains), Niger, Nigeria, Spain (imported), France (2 strains [1 imported]), and the United Kingdom (imported) (Figure 2). Again, patient isolate Gb100 and soil isolate C2 are most closely related to ST879. Soil isolate D50 grouped together with 3 STs representing 4 *B. thailandensis* strains isolated from Kenya, France, the United States, and Cambodia. Using this approach, we showed that the closest relatives of the strain that infected and eventually killed the patient reported here were ST879 and the strain isolated from soil around her community. Our whole-genome sequencing sample data have been submitted to a project that is undertaking whole-genome sequencing on a large number of *B. pseudomallei* isolates from around the world. This approach is anticipated to offer superior resolution of the global phylogeny of *B. pseudomallei* (9).

Discussion

We detected a case of melioidosis in a human in central Africa, confirmed the presence of *B. pseudomallei* in the environment in Gabon, and isolated *B. thailandensis* from an environmental sample from that part of the world. The low rate of antibody seropositivity among healthy children combined with the low prevalence of *B. pseudomallei* cultured from blood of patients in a local hospital, however, suggest that melioidosis is rare in this setting.

Only 4 of the 13 melioidosis cases acquired by humans in Africa and reported in the literature have been PCR

confirmed (9–12,29–34). We show with phylogenetic analysis that the newly identified patient isolate Gb100 groups with a *B. pseudomallei* isolate from a patient from Spain who had traveled across West Africa and Madagascar (12). *B. pseudomallei* seropositivity was reported during a World Health Organization investigation into an outbreak of severe pneumonia in the northeastern of the Democratic Republic of Congo (Eric Bertherat, pers. comm.) (35). However, in that study some of the *B. pseudomallei*–seropositive cases diagnosed as melioidosis were later diagnosed as plague, calling into question the value of serology-based testing in this setting (35). The predominant soil type at the sites from which *B. pseudomallei* was isolated was similar to the soil type from which *B. pseudomallei* strains were isolated in Cambodia (26,36). The low rate of *B. pseudomallei* positivity per site points toward a relatively low abundance of *B. pseudomallei* in Gabon soil when compared with highly melioidosis-endemic areas in Southeast Asia and Australia (21,37). The true distribution of melioidosis in Africa remains uncertain, but we now can expand this area toward the central African country of Gabon.

The genus *Burkholderia* comprises >30 species, of which *B. pseudomallei* and *B. mallei* are considered the most pathogenic (2,38). The isolation of *B. thailandensis* from soil in Gabon extends our knowledge of the geographic distribution of this species. This strain was positive by Bps latex agglutination; this finding is in agreement with previous findings of a *B. thailandensis* strain from Thailand with a Bps-like capsular polysaccharide variant that also had a positive Bps latex-agglutination result (39). Our phylogenetic analysis shows a divergence between the strain from Gabon and the original *B. thailandensis* E264 from Thailand, which is the most studied strain (4,5). Evidence of the presence of this bacterium in Africa will have implications for bacterial identification in clinical laboratories, diagnostic serology assays, and environmental studies.

Our study has several limitations. *B. pseudomallei* serology can be misleading; false-positive results are a major concern (40). Clearly, for assessing exposure to *B. pseudomallei*, an accurate, inexpensive, simple serologic assay is needed. In the interim, however, serologic evidence of exposure should be based on assays with known sensitivity and specificity against culture-confirmed melioidosis, and, to our understanding, the IHA is the best test for identifying melioidosis cases. Given the nature of working in a resource-poor environment, only limited information is available on the patient reported here (e.g., no imaging was performed to investigate the presence of deeper abscesses). With regard to the environmental study, *B. pseudomallei* is known for its capacity to survive in water and has been reported to be present in the air during severe weather (17); we, however did not investigate its presence in water and air in Gabon in this study. Furthermore, we cannot

dismiss the possibility of error during soil sampling although guidelines for environmental sampling of *B. pseudomallei* were followed (17).

In summary, we identified *B. pseudomallei* and *B. thailandensis* in the Gabon environment and discovered a novel *B. pseudomallei* ST that can cause lethal septic shock. *B. pseudomallei* is probably an underrecognized cause of disease in central Africa. We propose that melioidosis occurs in central Africa but that it is unrecognized because of the lack of diagnostic microbiology facilities.

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etymologia

Glanders [glan'dærz]

From the Old French *glandres* (“glands”) describing the enlargement of the parotid or submaxillary lymph nodes that is pathognomonic of the disease, glanders is a contagious disease of horses. Glanders is caused by *Burkholderia mallei* and is communicable to humans but should not be confused with human melioidosis, caused by *Burkholderia pseudomallei*. The chronic, cutaneous form of glanders presents as ulcerated skin lesions along major lymph and blood vessels and is known as farcy (from the Latin *farcire*, “sausage”). Among the first descriptions of glanders is in

the writings of Aristotle: “The ass suffers chiefly from one particular disease which they call ‘melis.’” In later writings, “melis” became “malleus,” which became a generic term for epizootics. Glanders has been eliminated in many industrialized countries, including in the United States, where there have been no naturally acquired human or animal cases since World War II. More recently, glanders has been reemerging in parts of the world; since 2000, outbreaks in horses have been reported in North Africa, the Middle East, and other areas.

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Protocol for Metagenomic Virus Detection in Clinical Specimens¹

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Sixty percent of emerging viruses have a zoonotic origin, making transmission from animals a major threat to public health. Prompt identification and analysis of these pathogens are indispensable to taking action toward prevention and protection of the affected population. We quantifiably compared classical and modern approaches of virus purification and enrichment in theory and experiments. Eventually, we established an unbiased protocol for detection of known and novel emerging viruses from organ tissues (tissue-based universal virus detection for viral metagenomics [TUViD-VM]). The final TUViD-VM protocol was extensively validated by using real-time PCR and next-generation sequencing. We could increase the amount of detectable virus nucleic acids and improved the detection of viruses $\leq 75,000$ -fold compared with other tested approaches. This TUViD-VM protocol can be used in metagenomic and virome studies to increase the likelihood of detecting viruses from any biological source.

Viruses responsible for disease outbreaks in humans naturally emerge either from the human population or as zoonoses by transmission from animal hosts (1). Viruses can also emerge unnaturally, either directly (e.g., bioterrorist attacks) or accidentally (e.g., laboratory infections). Despite these possibilities of virus emergence, 60% of emerging viruses have a zoonotic origin, thus highlighting transmission from animals to humans as a major threat to public health (2). Whenever viruses emerge, prompt identification of the agent and implementation of control measures to contain the outbreak are required.

Currently, various next-generation sequencing (NGS) approaches provide solutions for detection of purified and concentrated viruses (i.e., from cell culture). However, for clinical specimens, such as blood, other fluids, or infected organ tissues, successful detection of viruses is less likely because virus-to-host genome ratios are insufficient (3–6). Use of tissues from persons with suspected infections for virus detection enables elucidation of infection directly at the site of viral replication. Although detecting viruses

directly from infected organ tissue provides obvious and valuable advantages, reliable purification of viruses directly from tissues still remains a challenge.

In this study, we quantifiably and extensively compared classical and modern experimental approaches for virus purification and enrichment to finalize a protocol for unbiased detection of emerging viruses directly from organ tissues (tissue-based unbiased virus detection for viral metagenomics [TUViD-VM]) for an increased signal-to-noise ratio (ratio of virus genome to host genome) in virus detection. Use of this approach will reduce the amount of host nucleic acids required and save money and time in preparation of samples for NGS and the subsequent bioinformatic analysis.

Materials and Methods

We first describe how the protocol was developed and evaluated. We then describe the final virus purification and enrichment TUViD-VM protocol for metagenomic deep sequencing for nucleic acid from organ tissue (Figure 1).

Protocol Development

Ethics Statement

All procedures regarding the marmoset used in this study were performed in accordance with the European Association of Zoos and Aquaria Husbandry Guidelines for Callitrichidae, 2nd ed., 2010 (http://www.marmosetcare.com/downloads/EAZA_HusbandryGuidelines.pdf), which promotes the highest possible standard for husbandry of zoo animals. The marmoset was kept in Zoo Heidelberg (Heidelberg, Germany) with other marmosets in a species-appropriate environment enriched with material for occupation and activity and adequate feeding regimens 3 times a day. The marmoset that was euthanized did not have any additional signs of illness or infection. The production of specific pathogen-free eggs (VALO BioMedia GmbH, Osterholz-Scharmbeck, Germany) was performed in

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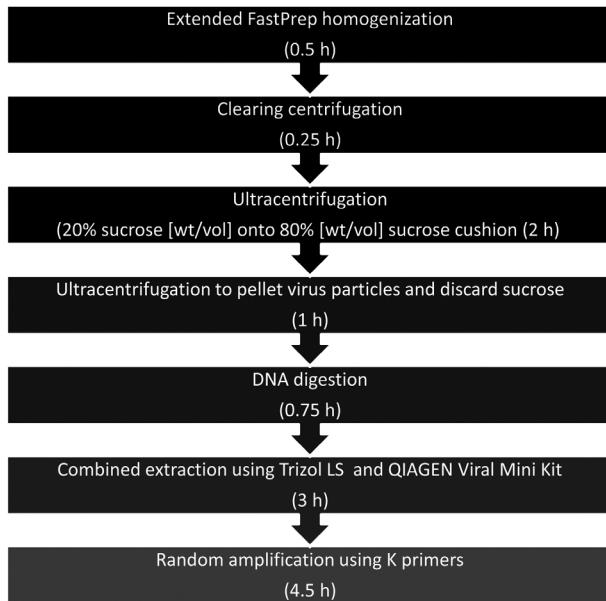


Figure 1. Schematic description of tissue-based universal virus detection for viral metagenomics protocol. Estimated durations of each step are shown in parentheses. The protocol takes 12 h to complete.

accordance with guidelines of the European Pharmacopoeia (EP7.0.5.2.2) and the US Department of Agriculture Veterinary Services (Memorandum 800.65).

All procedures regarding embryonated chicken eggs were based on German Animal Protection Laws. For infection, fertilized chicken eggs at embryonation day 11 were inoculated with virus into the allantois sack or onto the chorioallantoic membrane. Development of embryos was terminated at day 17 of embryonation by cooling the eggs overnight at 4°C. No further specific approval is needed for experiments on embryonated avians before time of hatching. However, additional approval from the internal ethics advisory board of the Robert Koch Institute was obtained and is available on request.

Study Design

To compare classical and modern experimental approaches of virus purification and enrichment, we designed a tissue model for internal organs of chicken, each infected with 1 of 4 viruses (poxvirus [vaccinia virus], reovirus [orthoreovirus], orthomyxovirus [influenza virus], and paramyxovirus [Sendai virus]) at low concentrations (Table 1; Table 2, <http://wwnc.cdc.gov/EID/article/21/1/14-0766-T2.htm>; online Technical Appendix, <http://wwnc.cdc.gov/EID/article/21/1/14-0766-Techapp1.pdf>). Viruses were chosen on the basis of their role in emerging zoonotic diseases and their morphologic and molecular heterogeneity to obtain results for a broad range of viruses (Table 3).

Model Tissue and Protocol Development

To establish a model tissue, we inoculated specific pathogen-free embryonated chicken eggs with 1 of the prechosen viruses at different concentrations. A detailed description of egg infection and preparation of the model tissue is shown in the online Technical Appendix. Reovirus (T3/Bat/Germany/342/08) (11) was chosen to represent a non-enveloped virus, orthomyxovirus (influenza A PR/8/1934) and paramyxovirus (Sendai virus) were chosen to represent enveloped viruses with an RNA genome, and poxvirus (vaccinia virus) was chosen to represent an enveloped virus with a DNA genome (Table 3). Viruses in this study were selected to optimize detection of viral zoonotic emerging diseases and possible virus bioterrorism agents.

To validate the model tissue homogeneity, we selected every ninth sample for simultaneous RNA/DNA extraction and determined copy numbers for all 4 viruses and the *gALTBP* gene (Figure 2). Samples showed an even Gaussian distribution of virus nucleic acids per aliquot and were considered suitable for subsequent experiments.

To establish a protocol for the purification and detection of unknown viruses from animal tissue, we tested different purification techniques and their combinations, including mechanical, enzymatic, and molecular biological methods; the main aim was to eliminate as much host DNA/RNA and maintain as much virus RNA/DNA as possible to optimize random PCR amplification of unknown viruses. The novel established protocol was tested to detect any virus from lung tissue derived from a New World monkey (marmoset), which had to be euthanized because of the unknown disease-causing agent.

We compared different techniques of virus purification, enrichment, and amplification (detailed description of methods compared is shown in the online Technical Appendix). In addition, complex purification techniques (digestion and ultracentrifugation) were compared by conducting experiments that had specific control factors (e.g., ultracentrifugation with different concentrations of sucrose, time and speed) (12). Organization of combinations of different control factors and their variable factors (e.g., concentration levels, duration or speed in orthogonal assays) enables conducting a minimal number of experiments. On the basis of results of all purification techniques, we developed a combined protocol to provide the maximized yield of virus RNA/DNA after purification.

Validation and Analysis of Methods Compared

All compared methods were analyzed simultaneously. Because evaluation of sample quality was ongoing, to exclude any extraction bias, an additional unprocessed control aliquot was extracted and measured with every batch. All results of 1 extraction were rigorously compared with a related control aliquot to normalize any

Table 1. Comparison of methods used to develop a protocol for metagenomic virus detection in infectious disease settings*

Purpose, method and supplier	Score†
Virus release/homogenization	
Ultrasonic (Sonopuls; Bandelin Electronic, Berlin, Germany)	+2
Dounce homogenizer (Kleinfeld Labortechnik, Gehrden, Germany)	+1
Qiashredder (QIAGEN, Hilden, Germany)	0
Trypsin (Life Technologies, Darmstadt, Germany)	+3
FastPrep Homogenizer (MP Biomedicals, Strasbourg, France) (longer homogenization time)	+4
Enrichment of virus particles	
Filtration 0.2-µm filter (Merck-Millipore, Temecula, CA, USA)	+4
Filtration 0.45-µm filter (Merck-Millipore)	-2
Fractionated filtration	-1
Durapore polyvinylidene fluoride filter tubes (Merck-Millipore)	+2
With or without clearing centrifugation	+3
Taguchi-optimized centrifugation: 20% sucrose cushion overlaying 80% sucrose cushion and second clearing ultracentrifugation	+4
PEG-It virus precipitation (System Biosciences, Mountain View, CA, USA)	+1
InRichment Virus Reagent Kit I (Analytik Jena AC, Jena, Germany)	-1
Digestion/removal of host nucleotides	
Turbo DNA-free (Ambion, Darmstadt, Germany) 30 min at 37°C with centrifugation	+4
RiboMinus Eukaryote Kit (Invitrogen Life Technologies, Grand Island, NY, USA)	+1
Nucleotide extraction	
QIAamp UltraSens Virus Kit (QIAGEN)	+2
QIAamp Viral RNA Mini Kit	+2
PureLink Viral RNA/DNA (Invitrogen Life Technologies)	+1
QIAamp MinElute Virus Spin Kit (QIAGEN)	-1
RTP DNA/RNA Virus Mini Kit (Invitex, Berlin, Germany)	-2
RTP DNA/RNA Virus Ultra Sense (Invitex)	0
NucleoSpin RNA II (Macherey Nagel, Dueren, Germany)	0
NucleoSpin DNA (Macherey Nagel)	+2
Phenol chloroform extraction (Carl Roth GmbH, Karlsruhe, Germany)	+3
TRIzol LS reagent (Life Technologies)	+4
Amplification	
N12 random primer	+3
N10 random primer	+2
WTA‡	+3
WGA	0
K primer‡ (7)	+3
3' locked random primer (8)	+1

*WTA, whole transcriptome amplification; WGA, whole genome amplification.

†For every relative quantification result that increased the ratio between host and virus nucleic acids, 1 point was assigned (maximum +4 points if the method led to a better detectability for all 4 viruses). For every decrease, 1 point was subtracted (minimum -4 points).

‡WTA and K primer showed similar results. However, when we considered the lower costs and ease of handling of K primers, we used K primers for this protocol.

variations caused by extraction, cDNA, and quantitative PCR (qPCR) performance.

Every result was evaluated for increasing the signal-to-noise ratio of virus to host-genome (this ratio is indicated by RQ). Given that $\Delta\Delta x = \Delta \text{measured} - \Delta \text{control}$, we assume that the ratio change between virus nucleic acids and host genome is given by $\Delta\Delta C_t = \Delta \text{purified} - \Delta \text{unprocessed}$, where C_t is the cycle threshold. To visualize relative quantification (RQ), $RQ (2 - \Delta\Delta C_t)$ was plotted against the respective methods. The RQ value indicates the x -fold change compared with that of the control aliquot (e.g., RQ value of 10 means a 10-fold higher Δ between virus and host genomes compared with the control aliquot) (13). Per definition of the RQ method, the area of significance lays outside RQ values of 0.5 and 2 if the samples show an even Gaussian distribution. Thus, results <0.5 and >2 were considered significant.

An additional scoring system was used to evaluate different methods. For every RQ result that increased the

ratio between host and virus nucleic acids, we gave 1 point (maximum +4 points if the method led to better detectability for all 4 viruses), and for every decrease, we subtracted 1 point (minimum is subsequently -4 points). Methods with the highest scores were chosen for establishment of a combined protocol that included purification of unknown viruses from any tissue source (Table 1).

Final TUViD-VM Protocol for the Enrichment and Purification of Viruses from Organ Tissue

Tissue Homogenate

For homogenization, a small cube of tissue (0.5–1 cm³) was placed in an autoclaved screw-cap tube (Sarstedt, Hildesheim, Germany) containing 1 mL of phosphate-buffered saline (PBS) buffer and 20–30 sterile ceramic beads. Tissue was disrupted by shaking 4 times at maximum speed at intervals of 15 s by using the FastPrep-24 Instrument

Table 3. Properties of 4 viruses used to develop a protocol for metagenomic virus detection in infectious disease settings*

Property	<i>Reovirinae</i> , reovirus	<i>Orthomyxovirinae</i> , influenza virus A	<i>Poxvirinae</i> , vaccinia virus	<i>Paramyxovirinae</i> , Sendai virus
Size, nm, shape	75–85, icosahedral	80–120, spherical, pleomorphic	270 × 350, brick-shaped complex	150–350, spherical, pleomorphic
Buoyant density, g/mL	1.36	1.2	1.23–1.27	1.31
Size genome, kbp	≈23.5	≈13.5	186–192	≈15.5
RNA/DNA	dsRNA	(–) ssRNA	dsDNA	(–) ssRNA
Genome organization	Linear, 10 segments	Linear, 8 segments	Linear, continuous	Linear, continuous
Envelope	No	Yes	Yes	Yes
Replication	Cytoplasm	Nucleus	Cytoplasm	Cytoplasm
Virion assembly	Cytoplasmic inclusion bodies (viral factories)	Cytoplasm	Cytoplasmic factory areas	Cytoplasm
Release	After virus-induced cell death	Budding from cell membrane	Exocytosis, cell lysis	Budding from cell membrane
Sensitivity	Unknown	Cesium chloride, heat, formaldehyde, SDS, ultraviolet light, oxidation compound	Unknown	Cesium chloride, heat, formaldehyde, SDS, oxidation compound

*Virus data were obtained from King et al. (9) and Tidona and Darai (10). –, negative. SDS, sodium dodecyl sulfate.

(MP Biomedicals, Strasbourg, France). The duration of this procedure was ≈0.5 h.

Clearing Centrifugation

A total of 200 mL of homogenate was placed in a 1.5-mL tube and vortexed vigorously. The homogenate was centrifuged for 5 min at 2,000 rpm in a bench top centrifuge (Eppendorf, Hamburg, Germany). The supernatant (≈170 mL) was transferred into a clean tube, and the pellet was discarded. The duration of this procedure was ≈0.25 h.

Ultracentrifugation for Virus Particle Separation

A total of 250 mL of 80% (wt/vol) sucrose solution was pipetted into a 2 3/8-in PA ultracentrifuge tube (Beckman Coulter, Krefeld, Germany) and gently overlaid with ≈3 mL of 20% (wt/vol) sucrose solution. The visibility of the phase interface between the 80% and 20% sucrose solutions was checked. The sucrose solution was gently overlaid with cleared tissue supernatant, and PBS was then added to the tubes. The tubes were centrifuged in an SW60 rotor (Beckman Coulter) at 30,000 rpm for 2 h at 4°C. The duration of this procedure was ≈2 h.

Ultracentrifugation to Pellet Virus Particles

The layer on the interface between the 20% and 80% sucrose solutions was collected and transferred into a 3 1/2-in tube (suitable for Beckmann SW32Ti rotors; Beckman Coulter). The collected layer was resuspended in ≈40 mL of PBS and mixed gently by pipetting up and down. The suspension was centrifuged for 1 h at 20,000 rpm and 4°C. The supernatant was then discarded. The duration of this procedure was ≈1 h. As an alternative method, virus particles can be precipitated overnight by using Peg-It (System Biosciences, Mountain View, CA, USA).

DNA Digestion

The pellet was resuspended in 245 mL of 1× digestion buffer (Turbo DNA Free Kit; Ambion, Darmstadt, Germany).

A total of Add 5 mL of Turbo DNase (Turbo DNA Free Kit; Ambion) was added and incubated for 30 min at 37°C. The suspension was transferred to a 1.5-mL reaction tube. A total of 10 mL of stop reagent (Turbo DNA Free Kit; Ambion) was added, incubated at room temperature for 1 min, and centrifuged at 2,000 rpm for 3 min. The supernatant was transferred to another tube, and pellet was discarded. The duration of this procedure was ≈0.75 h.

Combined TRIzol LS Extraction

A total of 750 mL of TRIzol LS (Invitrogen Life Technologies, Grand Island, NY, USA) was added to ≈250 mL of supernatant from previous procedures and homogenized by pipetting up and down 10 times. The mixture was incubated for 5 min at room temperature and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to precentrifuged phase-lock gel tube (5-Prime, Hilden, Germany). A total of 200 mL of chloroform–isoamyl alcohol was added and mixed by inverting the tube vigorously. The tube was incubated for 15 min at room temperature and centrifuged at 12,000 rpm for 15 min.

Approximately 280 mL of supernatant from the phase-lock gel tube was transferred to another tube containing 1,120 mL of AVL lysis buffer without carrier RNA (Viral RNA Mini Kit; QIAGEN, Hilden, Germany). A total of 700 mL of absolute ethanol was added and mixed by pulse vortexing. The solution was transferred in 600-mL portions to a QIAamp Mini Column, QIAGEN), centrifuged 8,000 rpm for 1 min, and the filtrate was discarded. The column was placed in a new collection tube, loaded again, and centrifuged until the lysate was added to the column. A total of 500 mL of 70% (wt/vol) ethanol was added and the column was centrifuged at 8,000 rpm for 3 min.

A mixture of 10 mL of DNase and 70 μL of RDD buffer (RNase-Free DNase Set; QIAGEN) was added to the column and incubated for 15 min at room temperature, as described by the manufacturer. The column was washed

with 500 mL of AW1 buffer, centrifuged at 8,000 rpm for 1 min, and the filtrate was discarded. The column was placed in a new tube, 500 mL of AW2 buffer was added, the tube was centrifuged at maximum speed for 3 min, and the filtrate was discarded. The column was then placed in a new tube, and the tube was centrifuged at maximum speed for 1 min to dry the column. A total of 30 mL of elution buffer was added to the column, incubated for 5 min at room temperature, and the column was centrifuged in a new 1.5-mL tube. A total of 30 mL of elution buffer was added to the column, incubated for 5 min at room temperature, and centrifuged in the same tube. RNA (≈ 60 mL) was chilled on ice. The duration of this procedure was ≈ 3 h.

Random Amplification

Single-stranded cDNA was produced by using the Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA, USA) and adapted for a 50-mL reaction containing 30 mL of RNA, 2 mL (40 μ mol/L) of K8N random primer (7), 3.2 mL (25 mmol/L) of dNTPs, 4 mL 10 \times buffer, 9 mL (50 mmol/L) of MgCl₂, 0.8 mL of RNase inhibitor, 0.6 mL of reverse transcriptase, and 0.4 mL of water). A total of 2 mL of K8N random primers and 3.2 mL of dNTPs were added to the 30 mL of RNA and heated at 95°C for 5 min before quenching on ice. The remaining contents of the mixture were heated at 42°C for 60 min before the enzyme was inactivated at 95°C for 10 min.

Double-stranded cDNA was produced by mixing 2 mL of K8N random primers, 3 mL of Klenow buffer (New England Biolabs, Ipswich, MA, USA), and 2 mL (2.5 mmol/L) of dNTPs with 19 mL of cDNA. The mixture was

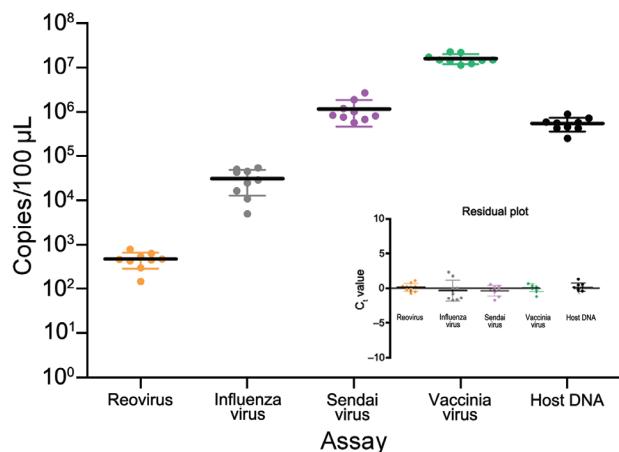


Figure 2. Validation of test aliquots of infected mode used for development of tissue-based universal virus detection for viral metagenomics protocol. Every ninth aliquot was extracted, and viral copy numbers were determined by using a quantitative PCR. SDs (error bars), medians (solid horizontal lines), and residual plots indicate homogeneity and mixture of test specimens. C_t, cycle threshold.

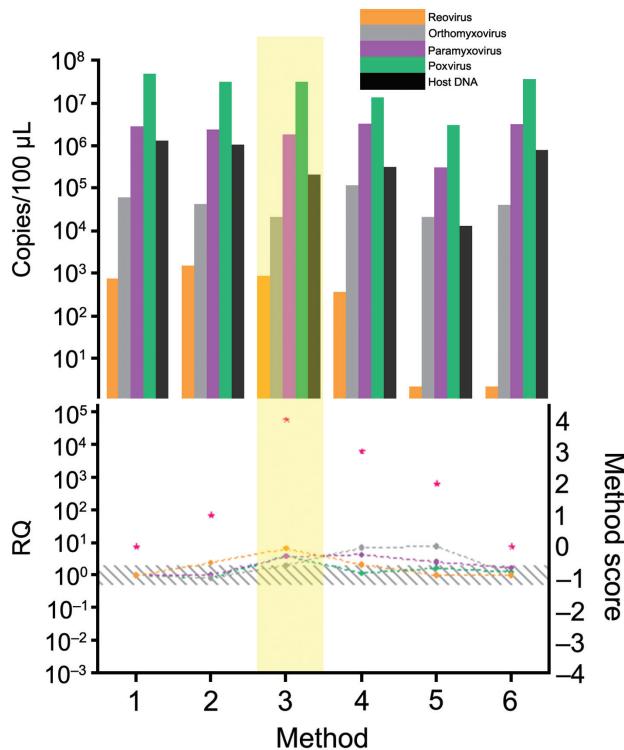


Figure 3. Comparison of tissue homogenization methods used for development of tissue-based universal virus detection for viral metagenomics protocol. Copy numbers were measured by quantitative PCR in duplicate. RQ, relative quantification: $RQ = 2^{-\Delta\Delta C_t}$; ($\Delta\Delta C_t = \Delta$ purified $- \Delta$ unprocessed). Lower panel left y-axis indicates signal-to-noise ratio (RQ) for all viruses tested. The method with the highest score was used to establish the protocol and is shaded in yellow. Red stars indicate highest scores. Diagonally striped area indicates not significant. C_t, cycle threshold. Numbers along baseline indicate method used: 1, control; 2, Dounce homogenizer; 3, extended homogenization; 4, trypsin; 5, ultrasound; 6, QIAshredder (QIAGEN, Hilden, Germany).

heated at 95°C for 2 min and cooled to 4°C. A total of 1.67 mL of Klenow fragment (New England Biolabs) was added and the mixture was at 37°C for 60 min. Double-stranded cDNA was purified by using the MSB Spin PCRapace Purification Kit (Invitex, Berlin, Germany) and an elution volume of 30 mL. Random amplification was performed by using the procedures reported by Stang and Korn (7). Successful random amplification (a 200–2,000-bp smear) was visualized by agarose gel electrophoresis of 10 mL of PCR product. The duration of this procedure was ≈ 4.5 h. Sequence information can be obtained by either cloning into sequencing vectors or by NGS.

NGS

RNA samples were fragmented by using the NEBNext Magnesium RNA Fragmentation Module (New England

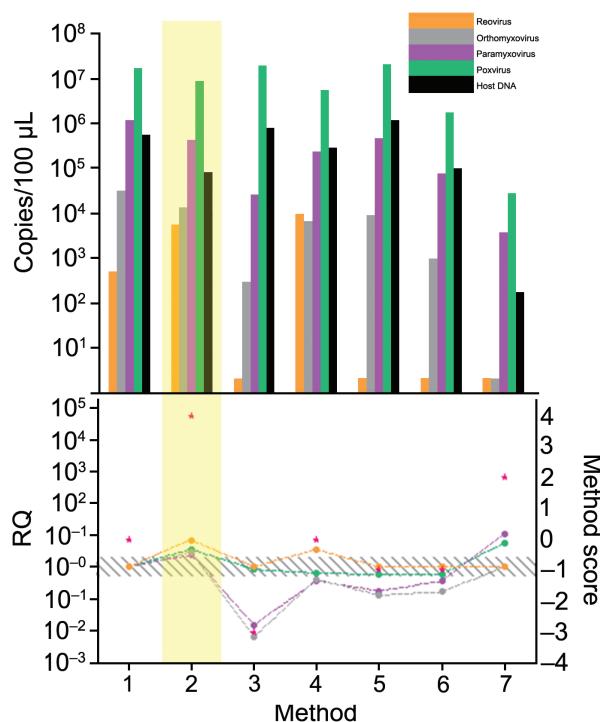


Figure 4. Comparison of filtration methods used for development of tissue-based universal virus detection for viral metagenomics protocol. Copy numbers were measured by quantitative PCR in duplicate. RQ, relative quantification: $RQ(2^{-\Delta\Delta C_t})$; ($\Delta\Delta C_t = \Delta$ purified $-\Delta$ unprocessed). Lower panel left y-axis indicates signal-to-noise ratio (RQ) for all viruses tested. The method with the highest score was used to establish the protocol and is shaded in yellow. Red stars indicate highest scores. Diagonally striped area indicates not significant. C_t , cycle threshold. Numbers along baseline indicate method used: 1, control; 2, 0.22- μ m filter; 3, 0.45- μ m filter; 4, filter extraction 1; 5, filter extraction 2; 6, fractionated filtration; 7, filter tubes.

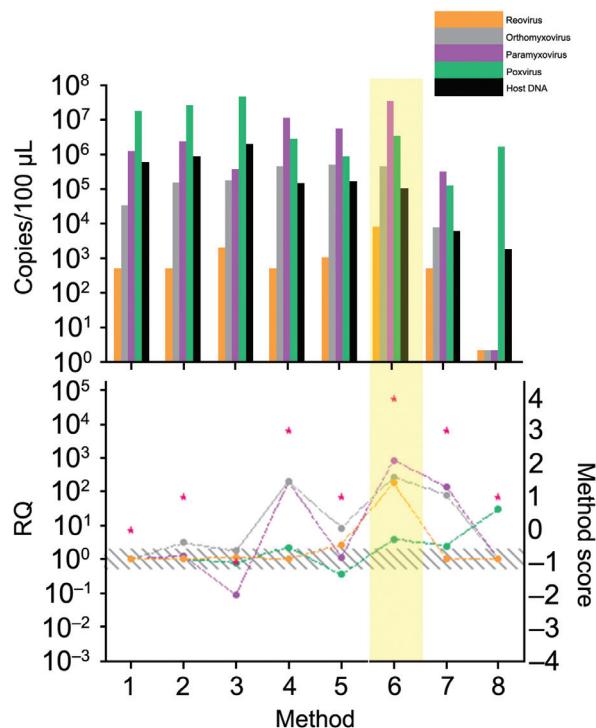


Figure 5. Comparison of enrichment methods used for development of tissue-based universal virus detection for viral metagenomics protocol. Copy numbers were measured by quantitative PCR in duplicate. RQ, relative quantification: $RQ(2^{-\Delta\Delta C_t})$; ($\Delta\Delta C_t = \Delta$ purified $-\Delta$ unprocessed). Lower panel left y-axis indicates signal-to-noise ratio (RQ) for all viruses tested. The method with the highest score was used to establish the protocol and is shaded in yellow. Red stars indicate highest scores. Diagonally striped area indicates not significant. C_t , cycle threshold. Numbers along baseline indicate method used: 1, control; 2, PEG-It (System Biosciences, Mountain View, CA, USA); 3, InRichment Virus Reagent Kit (Analytik Jena AC, Jena, Germany); 4, clearing centrifugation; 5, clearing centrifugation at 25,000 rpm for 2 h; 6, second clearing centrifugation after 20% sucrose centrifugation; 7, tissue enrichment; 8, Ribominus Eukaryote Kit (Life Technologies, Grand Island, NY, USA).

Biolabs). RNA was purified by using RNeasy MinElute (QIAGEN). For cDNA synthesis, Superscript II and Murine RNase inhibitor (New England Biolabs) were used. Second-strand synthesis was performed by using the NEBNext mRNA Second Strand Synthesis Module (New England Biolabs) and purified by using the MinElute PCR Purification Kit (QIAGEN).

Double-stranded cDNA, DNA, and random PCR products were quantified by using the Qubit HS dsDNA Kit (Invitrogen Life Technologies). Sequencing libraries were established by Ion Xpress Plus Fragment Library Kit (without chemical fragmentation) with indices (Ion Xpress Barcode Adapters 1–16 Kit). The sequencing library was then amplified by using an emulsion-based clonal amplification PCR in the Ion OneTouch 200 Template v2 DL Kit and enriched by using an Ion OneTouch Enrichment System. Sequencing was performed on an IonTorrent PGM

in the Ion PGM Sequencing 300 Kit with the Ion 318 Chip Kit (Invitrogen Life Technologies).

NGS Data Analysis

Programs used for sequence analysis were Geneious Pro R6 (Biomatters, Auckland, New Zealand) and Bowtie2align (14). The percentage of bases ($Q>20$) was $\approx 80\%$ before length filtering (100–1,000 nt) was applied to remove shorter reads. No additional quality trimming was applied because the quality average was sufficient for our approach. Remaining reads were mapped to the whole reference genomes (or all segments of reference genome) by using Bowtie2align for paramyxovirus (Sendai virus strain Tianjin; GenBank accession no. EF679198), reovirus (T3/Bat/Germany/342/08, 10 segments; JQ412755–JQ412764),

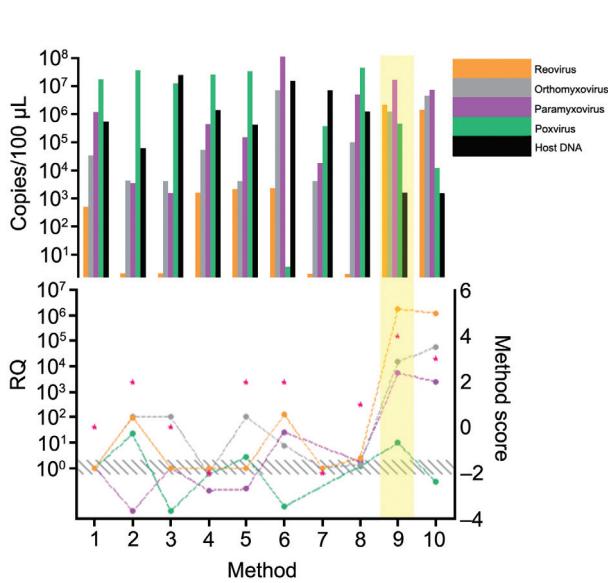


Figure 6. Comparison of extraction methods used for development of tissue-based universal virus detection for viral metagenomics protocol. Copy numbers were measured by quantitative PCR in duplicate. RQ, relative quantification: $RQ (2^{-\Delta\Delta C_t})$; ($\Delta\Delta C_t = \Delta C_t \text{ purified} - \Delta C_t \text{ unprocessed}$). Lower panel left y-axis indicates signal-to-noise ratio (RQ) for all viruses tested. The method with the highest score was used to establish the protocol and is shaded in yellow. Red stars indicate highest scores. Diagonally striped area indicates not significant. C_t , cycle threshold. Numbers along baseline indicate method used: 1, Nucleospin RNA II (Macherey Nagel, Dueren, Germany); 2, Nucleospin DNA (Macherey Nagel); 3, RTP DNA/RNA Virus Ultra Sense (Invitex, Berlin Germany); 4, RTP DNA/RNA Virus Mini Kit (Invitex); 5, QIAamp UltraSens Virus Kit (QIAGEN, Hilden, Germany); 6, QIAamp Viral RNA Mini Kit (QIAGEN); 7, QIAamp MinElute Virus Spin Kit (QIAGEN); 8, PureLink Viral RNA/DNA (Invitrogen Life Technologies, Grand Island, NY, USA); 9, TRIzol LS; 10, phenol chloroform.

orthomyxovirus (influenza H1N1 strain A/Puerto Rico 8-SV14/1934, 8 segments; CY040170-CY040177), and poxvirus (vaccinia virus strain WR, no. AY243312). Coverage of genomes was calculated in weighted average for segmented genomes.

Results

Development of Protocol

Every step of the TUViD protocol (homogenization of tissue, filtration, digestion, enrichment, extraction, and random amplification) was compared with alternative approaches. Results are shown in Figures 3–7. Each approach was tested with individual samples, which were measured by using 5 PCRs specific for viruses used and host background in 2 replicates (10 reactions/sample): Results were quantified and evaluated in qPCRs for the 4 viruses and presence of host nucleic acids (online Technical Appendix; Table 4,

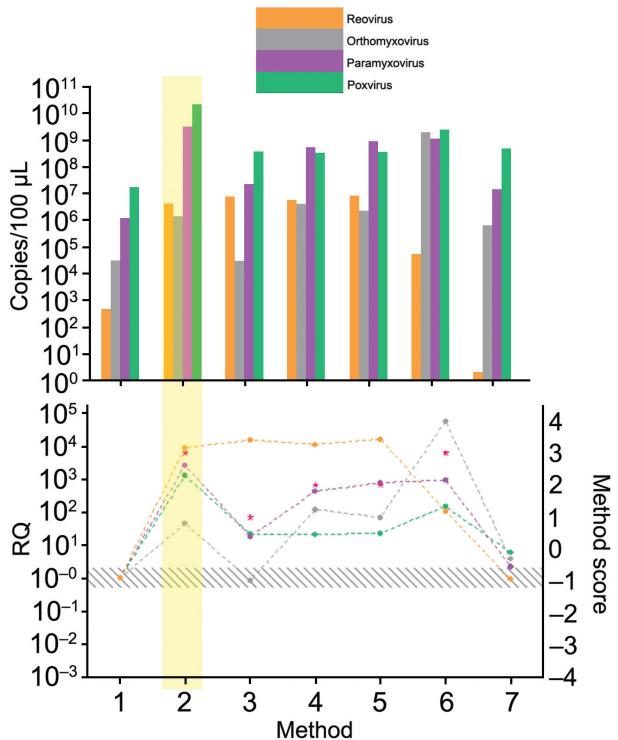


Figure 7. Comparison of primers and random amplification methods used for development of tissue-based universal virus detection for viral metagenomics protocol. Copy numbers were measured by quantitative PCR in duplicate. RQ, relative quantification: $RQ (2^{-\Delta\Delta C_t})$; ($\Delta\Delta C_t = \Delta C_t \text{ purified} - \Delta C_t \text{ unprocessed}$). Lower panel left y-axis indicates signal-to-noise ratio (RQ) for all viruses tested. The method with the highest score was used to establish the protocol and is shaded in yellow. Red stars indicate highest scores. Diagonally striped area indicates not significant. C_t , cycle threshold. Numbers along baseline indicate method used: 1, control; 2, K primer; 3, 3' locked primer; 4, N12 primer; 5, N primer; 6, whole transcriptome amplification (QIAGEN, Hilden, Germany); 7, whole genome amplification (QIAGEN).

<http://wwwnc.cdc.gov/EID/article/21/1/14-0766-T4.htm>; Figures 3–7). A scoring system was developed to assess the optimal combination of all 4 viruses (Table 1; Figures 3–7). A preliminary protocol was further validated and adjusted until no host nucleic acids were detectable by qPCR. This protocol maximized the amount of amplified virus nucleic acids. Subsequently, we established an unbiased protocol for the detection of known and novel viruses in infected organ tissues (TUViD-VM).

TUViD-VM Validation by NGS

The TUViD-VM protocol was validated by NGS of 4 aliquots of the model tissue. One aliquot was prepared by using the TUViD-VM protocol developed in this study, and 3 aliquots were prepared by using other approaches

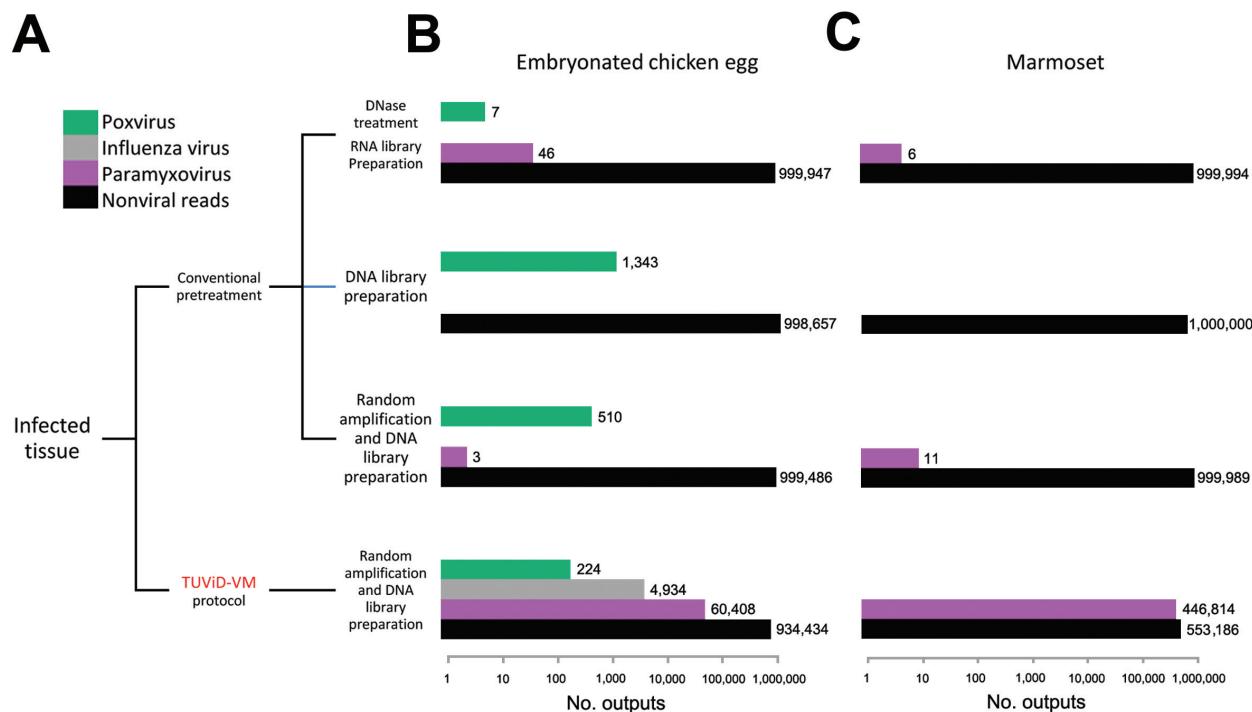


Figure 8. Results of comparative next-generation sequencing used for development of tissue tissue-based universal virus detection for viral metagenomics (TUViD-VM) protocol. A) Sample preparation flowchart to generate 4 next-generation sequencing approaches. B) Results obtained for model tissue (chicken) infected with 4 viruses: vaccinia virus (poxvirus) Sendai virus (paramyxovirus), influenza virus (A/PR8/1934), or reovirus (T3/Bat/G/342/08). The baseline is log-scaled, and normalized read numbers are indicated. C) Results of marmoset sample proof of principle, Sendai virus–infected lung tissue. The baseline is log-scaled, and normalized read numbers are indicated.

commonly used for unbiased virus detection (Figure 8; online Technical Appendix). We chose the Invitrogen Life Technologies platform because of its rapid run time and read length, which are crucial for diagnostic purposes. All independent runs were normalized to 1,000,000 output reads for reliable comparison (Table 5; Figure 8). NGS results confirmed the substantial increase in virus nucleic acids, as well as the decrease of host nucleic acids achieved by purification with the novel protocol. The amount of detectable virus nucleic acids was increased >1,000-fold compared with other NGS approaches (Figure 8). For example, although the best NGS approach delivered 40 reads for paramyxovirus in infected chicken tissue, the TUViD-VM protocol resulted in >60,000 reads (97.80% coverage of the complete genome) (Figure 8; Figure 9, <http://wwwnc.cdc.gov/EID/article/21/1/14-0766-F9.htm>; Figure 10, <http://wwwnc.cdc.gov/EID/article/21/1/14-0766-F10.htm>; Table 5).

To provide a proof of concept, we prepared lung tissue from the marmoset that was euthanized and had a natural respiratory infection with Sendai virus by using the 4 approaches and sequenced by using the Invitrogen Life Technologies protocol. Using the TUViD-VM protocol, we found that the amount of detectable virus in marmoset

tissue increased 75,000-fold compared with that for other NGS approaches (>400,000 Sendai virus reads compared with 6), which represented 99.98% coverage of the Sendai virus genome and ≈50% of the total read output (Figures 8, 10; Table 5).

Discussion

In this study, we successfully established a purification and enrichment protocol, which shows rapid and reliable results, for detection of known and novel viruses in tissues. Likelihood of detection of RNA viruses was increased. In addition, detection of DNA incorporated in virus particles was not affected even though DNA digestion was performed. The cutoff sensitivity was 100–1,000 virus copies/mL of homogenized organ material (e.g., reovirus; Table 5). The cutoff sensitivity of compared approaches was ≥10⁶ virus copies/mL. The TUViD-VM protocol (from solid tissue sampling to nucleic acid preparation for NGS) takes 12 h to complete. If one allows 16 h for NGS, the TUViD-VM protocol provides sequence data output within 28 h.

Current NGS techniques used for metagenomic approaches produce large amounts of sequence data, which might increase the likelihood of detection of diminutive amounts of virus in comparison with the host genome.

Table 5. Output of next-generation sequencing for development of a protocol for metagenomic virus detection in infectious disease settings*

Name	No. Original reads	No. remaining reads†	Minimum, maximum (mean) read length, nt†	Sendai virus	Vaccinia virus	Influenza virus, A/PR8/1934	Reovirus, T3/Bat/G/342/08	No. nonviral reads
Chicken RNA library	1,636,344	1,076,582	100, 464 (240)	46	7	0	0	999,947
Chicken DNA library	1,332,908	808,516	100, 463 (248)	0	1,343	0	0	998,657
Chicken random library	1,347,059	576,467	100, 460 (199)	3	510	0	0	999,486
Chicken TUViD-VM protocol	2,021,403	969,236	100, 455 (220)	60,408	224	4,934	0	934,434
Marmoset RNA library	2,859,201	1,555,567	100, 464 (223)	6	NA	NA	NA	999,994
Marmoset DNA library	2,856,326	1,711,121	100, 464 (246)	0	NA	NA	NA	1,000,000
Marmoset random library	598,451	355,443	100, 464 (200)	11	NA	NA	NA	999,989
Marmoset TUViD-VM protocol	1,007,051	640,088	100, 460 (223)	446,813	NA	NA	NA	553,186

*Standardization value for all procedures was 1,000,000. TUViD-VM, tissue-based universal virus detection for viral metagenomics; NA, not applicable.
†After length filtering of 100–1,000 nt.

The only limiting factor seems to be the cost required for processing 1 sample and capacities for computational analysis of results. This *in silico* analysis should increase the signal-to-noise ratio of relevant sequences by subtracting nonrelevant sequences, such as the host genome. However, genome sequence data for mammals are limited; only 23 sequences (0.4%) for 5,487 species (18). Just 3 genome sequences are available for bats, although they are the second most abundant mammalian species (exceeded only by rodents). There are >1,100 species of bats worldwide and they are suspected vectors of pathogenic viruses (e.g., Ebola virus, Nipah virus, Hendra virus, lyssavirus, and severe acute respiratory syndrome coronavirus). Thus, it seems inefficient to invest large amounts of time, money, and effort in obtaining large datasets, only to invest even more resources to categorize them. Furthermore, quantitative comparison of the virus-enrichment strategies described enables evaluation of multiple classical and modern approaches.

The TUViD-VM described protocol increases the signal-to-noise ratio by as much as 75,000-fold than that for compared approaches and can detect virus genomes quickly in infected tissues (Figures 9, 10). Although sequencing of nucleic acid from relatively pure sources (e.g., cell culture, allantoic fluids) is well established and results in reasonable output (11,19,20), sequencing of nucleic acid clinical specimens is still challenging. Other studies reported 0.1% to <10% mammalian virus reads from clinical samples, such as tissue, guano, feces, and pharyngeal swab specimens (3,19,21–24). A method reported by Daly et al. showed promising results for detection of DNA viruses but lacked similar results for detection of RNA viruses (25). In contrast, our protocol resulted in up to 45% mammalian RNA virus reads directly from infected organ tissue (Figure 8).

After its successful and extensive validation, we highly recommend this protocol for investigation of outbreaks with unknown viral etiologic agents in humans and animals. Furthermore, this protocol can be used in metagenomic virome studies and will be beneficial whenever library construction is necessary (i.e., molecular cloning and NGS) to increase detection likelihood for viruses from any biological source. This protocol would be particularly useful for increasing the signal-to-noise ratio in virus analysis of biological samples in which levels of background nucleic acids are high, which result in difficulties in virus detection and identification. Thus, the TUViD-VM protocol described greatly increases the likelihood of detecting viruses during outbreaks of emerging infectious diseases and in metagenomic virus detection studies.

Acknowledgments

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Neurotropic Threat Characterization of *Burkholderia pseudomallei* Strains

Jodie Morris, Anne Fane, Catherine Rush, Brenda Govan, Mark Mayo,
Bart J. Currie, and Natkunam Ketheesan

The death rate for neurologic melioidosis is high. Whether certain *Burkholderia pseudomallei* strains are more likely than other strains to cause central nervous system infection and whether route of infection influences the neurotropic threat remain unclear. Therefore, we compared the virulence and dissemination of Australian clinical isolates collected during October 1989–October 2012 from patients with neurologic and nonneurologic melioidosis after intranasal and subcutaneous infection of mice in an experimental model. We did not observe neurotropism as a unique characteristic of isolates from patients with neurologic melioidosis. Rather, a distinct subset of *B. pseudomallei* strains appear to have heightened pathogenic potential for rapid dissemination to multiple tissues, including the central nervous system, irrespective of the infection route. This finding has valuable public health ramifications for initiating appropriate and timely therapy after exposure to systemically invasive *B. pseudomallei* strains. Increasing understanding of *B. pseudomallei* pathology and its influencing factors will further reduce illness and death from this disease.

Melioidosis is caused by the gram-negative bacterium *Burkholderia pseudomallei*. It incorporates a wide spectrum of clinical disease that ranges from severe, rapidly fatal, invasive disease to asymptomatic latent infection; thus, diagnosis is immensely challenging (1). No vaccine against melioidosis is currently available. The ability of *B. pseudomallei* to cause severe, rapidly fatal, invasive infections and to persist in the environment for extended periods, plus its intrinsic resistance to many antibacterial drugs, make *B. pseudomallei* a desirable candidate for use as a bioterrorism agent (1). Furthermore, *B. pseudomallei* can invade host cells, including macrophages, neutrophils,

and other cells of the immune system, and persist within them (2,3). Without appropriate drug therapy, the death rate for melioidosis can exceed 90% (4,5).

Neurologic abnormalities occur in 3%–5% of melioidosis cases, and more than one quarter of those are fatal (5–8). Many similarities have been described regarding the clinical features of neurologic melioidosis in naturally infected animals and humans and in animal models infected with *B. pseudomallei* (6,9–15). Cranial nerve palsies and unilateral limb weakness are frequently described in patients with neurologic melioidosis (6,7,9,13,14). Flaccid paraparesis, commonly documented in animals with *B. pseudomallei* infection, also has been reported in humans (6,10,15). Often, microscopic and macroscopic abscesses are evident, and a predilection of *B. pseudomallei* for the brainstem and spinal cord has been suggested (9,12–14).

In contrast to patients with other forms of melioidosis, those with melioidosis with central nervous system (CNS) involvement are less likely to have predisposing risk factors, such as diabetes and chronic lung disease (6,8,9). Relatively little is known about the potential for different *B. pseudomallei* strains to cause severe disease, including whether particular strains are more likely to cause neurologic sequelae or whether CNS involvement is a consequence of the mode of delivery of *B. pseudomallei*. Neurologic melioidosis can result from direct invasion or through hematogenous spread (3,6,11,16,17). Initial suggestions that neurologic melioidosis might result from damage from immune or toxin-mediated mechanisms (6,12) has been supplanted by the recognition that direct invasion of brain and spinal cord by bacteria is evident on histologic examination of samples from case-patients who died (16). Furthermore, direct invasion of the brain by *B. pseudomallei* was recently demonstrated in an experimental model of melioidosis meningitis after delivery of intracellular bacteria by CNS-infiltrating CD11b⁺ immune cells (3).

Given the high rate of death from neurologic melioidosis, interest is increasing in improving understanding of its pathogenesis, particularly the potential for different *B.*

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pseudomallei isolates to cause neurologic melioidosis and the influence of the route of transmission on entry into and dissemination within the CNS. Therefore, using a well-characterized animal model of melioidosis (18) and clinical isolates of *B. pseudomallei* collected in the Northern Territory, Australia, during October 1989–October 2012 (7), we sought to determine whether strains isolated from patients with neurologic melioidosis (neurologic isolates) showed higher virulence levels and neurotropism than isolates from patients with nonneurologic melioidosis (nonneurologic isolates) after respiratory and percutaneous exposure.

Methods

B. pseudomallei Isolates

Eleven *B. pseudomallei* clinical isolates were analyzed. Six of these isolates were from patients with moderate to severe neurologic melioidosis, and 5 were from patients with nonneurologic melioidosis, both disseminated and localized cases (Table 1).

Animals

The C57BL/6–BALB/c mouse model is a well-characterized model of differential susceptibility to *B. pseudomallei* (18). We included C57BL/6 and BALB/c mice to enable comparison of disease progression within immunocompetent and immune-impaired hosts, respectively. We purchased 8- to 12-week-old BALB/c and C57BL/6 mice from the Small Animal Breeding Facility, James Cook University (Townsville, QLD, Australia). Experiments were conducted in accordance with National

Health and Medical Research Council guidelines and were approved by the institutional ethics committee (A1500).

Preparation and Delivery of *B. pseudomallei* Isolates

B. pseudomallei isolates were cultured in tryptic soy broth at 37°C to logarithmic phase. After washing in phosphate-buffered saline (PBS), pH 7.2, bacteria were suspended to 10⁸ CFU/mL. Serial 10-fold dilutions were prepared in sterile PBS to obtain required infectious doses. Doses were confirmed retrospectively by plating serial dilutions onto Ashdown agar (AA). To mimic natural routes of infection, intranasal or subcutaneous routes were used for inoculation with *B. pseudomallei* by previously described methods (18).

Determination of 50% Infectious Dose

Groups of 5 mice were inoculated intranasally and subcutaneously at 10-fold increasing doses of *B. pseudomallei*, ranging from 10⁰ CFU to 10⁷ CFU (18). Survival was monitored for 21 days, and moribund mice were euthanized. At necropsy, organs were observed for presence of visible abscesses. Spleens were homogenized and plated on AA to confirm the presence of *B. pseudomallei* in mice that died from their infection within the experiment period. Mice that survived to 21 days after infection were euthanized and underwent necropsy to determine whether abscesses were visible in spleen and liver. For mice infected subcutaneously, the subcutaneous adipose tissue at the site of infection was also assessed. Tissue homogenates were cultured on AA to confirm the presence or absence of persistent *B. pseudomallei* infection. We determined the 50% infectious dose (ID₅₀)

Table 1. Clinical features of *Burkholderia pseudomallei* strains isolated from patients with neurologic and nonneurologic melioidosis and their virulence in C57BL/6 and BALB/c mice, Northern Territory, Australia, October 1989–October 2012*

MSHR ID no.	Age, y/sex	Clinical feature	Risk factor	ID ₅₀ in mice (CFU)†			
				Intranasal		Subcutaneous	
				C57BL/6	BALB/c	C57BL/6	BALB/c
Neurologic							
668‡	53/M	Severe neurologic signs	None	4.1 × 10 ⁴	2.9 × 10 ²	7.1 × 10 ³	<10
305§	64/M	Severe neurologic signs	Alcohol use	2.6 × 10 ²	2.6 × 10 ²	3.7 × 10 ⁴	<10
62‡	24/M	Severe neurologic signs	None	2.2 × 10 ²	6.3 × 10 ¹	2.4 × 10 ²	<10
435‡	37/M	Severe neurologic signs	Kava	5.0 × 10 ²	1.3 × 10 ²	<10	<10
1153§	60/M	Severe neurologic signs	Diabetes mellitus	1.7	<10	<10	<10
3709‡	14/M	Moderate neurologic signs	None	4.4 × 10 ⁴	1.8 × 10 ⁴	2.2 × 10 ²	1.3 × 10 ²
Nonneurologic							
1655‡	61/F	Chronic pulmonary	Bronchiectasis	>10 ⁸	>10 ⁸	>2 × 10 ⁸	>2 × 10 ⁸
465§	67/M	Septicemia	Diabetes mellitus, chronic obstructive pulmonary disease	1.3 × 10 ⁵	1.1 × 10 ³	8.3 × 10 ⁵	<10
2138‡	49/F	Septicemia	Diabetes mellitus	3.6 × 10 ³	<10	4.2 × 10 ³	1.2 × 10 ¹
346‡	49/M	Chronic pulmonary	Alcohol use	8.5 × 10 ⁴	6.0 × 10 ⁴	6.0 × 10 ⁵	7.0 × 10 ⁵
543‡	22/F	Skin ulcer	None	2.9 × 10 ²	8.5 × 10 ¹	1.2 × 10 ¹	<10

*ID₅₀, 50% infectious dose; MSHR ID, neurologic isolate identification.

†ID₅₀ determined after intranasal and subcutaneous infection of C57BL/6 and BALB/c mice

‡Nonfatal.

§Fatal.

from the total number of mice that either died of their infection or had evidence of persistent infection 21 days postinfection (dpi) using a modified version of the Reed and Meunch method (19). ID₅₀ for neurologic and nonneurologic isolates are expressed as mean log₁₀ CFU ± the standard error of the mean (SEM). Virulence, as defined by the ID₅₀ of *B. pseudomallei* isolates derived from patients with neurologic and nonneurologic melioidosis, were compared in BALB/c and C57BL/6 mice after both intranasal and subcutaneous infection.

Determination of Bacterial Load

At specified time points, 5 mice were euthanized by cardiac puncture, and blood was collected into sterile tubes containing lithium heparin. Bacterial load in blood was determined by plating serial dilutions of whole blood in PBS onto AA and counting colonies after 24–48 h incubation at 37°C. Immediately after collection of blood, the liver, spleen, lung, lymph nodes (cervical and inguinal), brain, and nasal-associated lymphoid tissue were aseptically excised. Tissue bacterial load was determined by homogenizing tissue in 1 mL of PBS and plating serial dilutions onto AA for colony counts. The detection limit of bacteria in tissues was 2 CFU. Data were expressed as the mean log₁₀ CFU ± SEM.

Statistical Analysis

For statistical analysis, we used Graphpad Prism version 6 (<http://www.graphpad.com>). Kaplan–Meier survival curves were used to compare susceptibility to infection with *B. pseudomallei* isolates after infection by different routes. ID₅₀ for neurologic and nonneurologic *B. pseudomallei* isolates were compared by using Student *t* test. Bacterial loads in organs after *B. pseudomallei* infection by different routes were tested for significance using 1-way analysis of variance based on normally distributed sets of data. Comparisons were considered to be significant at $p \leq 0.05$.

Results

Although neurologic isolates tended to be more virulent (lower ID₅₀) than nonneurologic isolates after intranasal infection, this finding did not reach statistical significance (Table 1). Consistent with previous evidence for differential susceptibility, C57BL/6 mice demonstrated greater resistance than BALB/c mice to *B. pseudomallei* infection, as indicated by their 10-fold higher ID₅₀ (18).

Signs of CNS involvement (i.e., head tilt and/or circling, difficulty walking, limb paresis) developed after intranasal infection of BALB/c and C57BL/6 mice, typically 8–12 dpi. This feature was not unique to neurologic isolates; head tilt and limb paralysis also were observed in mice infected with nonneurologic isolates. The development of neurologic signs corresponded with bacterial loads

in brain, reaching $>10^3$ CFU. However, the data suggest that development of neurologic signs did not depend on the initial infectious dose because serial increases in the inoculating dose failed to cause a stepwise increase in number of C57BL/6 mice with neurologic signs (Table 2). Similar trends were observed after intranasal infection of BALB/c mice (data not shown).

Table 2. Development of signs of neurologic involvement* in C57BL/6 mice after intranasal infection with *Burkholderia pseudomallei* strains isolated from patients with neurologic and nonneurologic melioidosis, Northern Territory, Australia, October 1989–October 2012

MSHR ID no.†	Inoculating dose, CFU	No. mice with neurologic signs/total mice infected
Neurologic		
668	2.9×10^3	1/5
	2.9×10^4	0/5
	2.9×10^5	2/5
	2.9×10^6	2/5
305	2.6×10^3	0/5
	2.6×10^4	1/5
	2.6×10^5	3/5
	2.6×10^6	0/5
62	2.2×10^3	1/5
	2.2×10^4	1/5
	2.2×10^5	2/5
	2.2×10^6	1/5
435	3.0×10^3	0/5
	3.0×10^4	3/5
	3.0×10^5	2/5
	3.0×10^6	2/5
1153	5.3×10^1	1/5
	5.3×10^2	3/5
	5.3×10^3	0/5
	5.3×10^4	1/5
3709	2.2×10^3	0/5
	2.2×10^4	0/5
	2.2×10^5	0/5
	2.2×10^6	0/5
Nonneurologic		
1655	1.1×10^4	0/5
	1.1×10^5	0/5
	1.1×10^6	0/5
	1.1×10^7	0/5
465	6.6×10^3	0/5
	6.6×10^4	0/5
	6.6×10^5	2/6
	6.6×10^6	1/6
2138	2.4×10^2	0/5
	2.4×10^3	0/5
	2.4×10^4	1/5
	2.4×10^5	2/4
346	4.2×10^3	0/5
	4.2×10^4	0/5
	4.2×10^5	2/5
	4.2×10^6	0/5
543	9.4×10^2	1/5
	9.4×10^3	1/5
	9.4×10^4	3/5
	9.4×10^5	2/5

*Head tilt, difficulty walking, limb paresis.

†MSHR ID, neurologic isolate identification.

After subcutaneous infection with *B. pseudomallei* isolates, ID_{50} ranged from <10 CFU to $>2 \times 10^8$ CFU in BALB/c and C57BL/6 mice. Consistent with intranasal infection, BALB/c mice were more susceptible than C57BL/6 mice to subcutaneous infection with *B. pseudomallei*, as indicated by their 10–100-fold lower ID_{50} . Similar to findings after intranasal infection, neurologic isolates tended to be more virulent (lower ID_{50}) than nonneurologic isolates after subcutaneous infection, although this finding did not reach statistical significance.

Mean ID_{50} was comparable for C57BL/6 mice after intranasal (3.2×10^4 CFU) or subcutaneous (9.3×10^4 CFU) infection with neurologic isolates. Similarly, mean ID_{50} for BALB/c mice did not differ between intranasal (7.6×10^2 CFU) and subcutaneous (8.1×10^2 CFU) infection. Neurologic isolates appeared to be more infectious for BALB/c mice when delivered subcutaneously rather than intranasally ($p = 0.04$). However, we did not observe this phenomenon for C57BL/6 mice.

We conducted a second series of studies to compare early bacterial load kinetics within tissues after intranasal and subcutaneous infection with *B. pseudomallei*. The purpose of these studies was to determine whether infection of the brain occurred more rapidly after intranasal than subcutaneous infection with isolates from neurologic melioidosis. The neurologic isolates, MSHR435 and MSHR1153, were associated with severe neurologic melioidosis whereby the CNS was suspected to be the primary site of infection and therefore represent isolates with high potential for direct CNS invasion after intranasal exposure in an animal model. BALB/c mice were inoculated with equivalent doses of *B. pseudomallei* intranasally or subcutaneously. Survival was monitored for 21 days. In addition, bacterial loads were determined in nasal-associated lymphoid tissue, draining lymph nodes (cervical or inguinal), blood, lung, brain, spleen, liver, and subcutaneous adipose tissue at the site of infection at 1, 3, and 7 dpi. BALB/c mice tended to be more susceptible to MSHR435 when infected subcutaneously; the rate of death reached 100% within 18 days, although this finding was not statistically significant ($p = 0.29$) (Figure 1, panel A). However, the death rate for infection of BALB/c with MSHR1153 was significantly higher after subcutaneous inoculation; hind leg paresis developed within the second week after infection that necessitated euthanasia of 6 of the 10 mice ($p = 0.03$, Figure 1, panel B). In contrast to subcutaneous infection, greater variability in disease progression was associated with intranasal inoculation of mice with *B. pseudomallei*, ranging from rapid systemic dissemination in some mice to low-level persistence in the respiratory tract with potential for clearance within a week after exposure. Infection was established in 100% of mice when MSHR435 or MSHR1153 was delivered

subcutaneously. Consequently, the differences in overall death rates for mice after intranasal and subcutaneous infection reflect the variability in dissemination of *B. pseudomallei* after respiratory exposure.

Regardless of the route of infection, dissemination occurred rapidly; bacteria were detected not only at sites of infection but also in draining lymph nodes and spleen by 1 dpi (Figure 2, panels A and B). Bacterial loads continued to increase significantly from 3 dpi (Figure 2, panels C, D) through 7 dpi (Figure 2, panels E, F), with comparable levels in spleen, liver, and lung after intranasal or subcutaneous infection for MSHR435 and MSHR1153. Bacterial loads in the brain of mice infected with MSHR435 and MSHR1153 were low or undetectable within the first week after

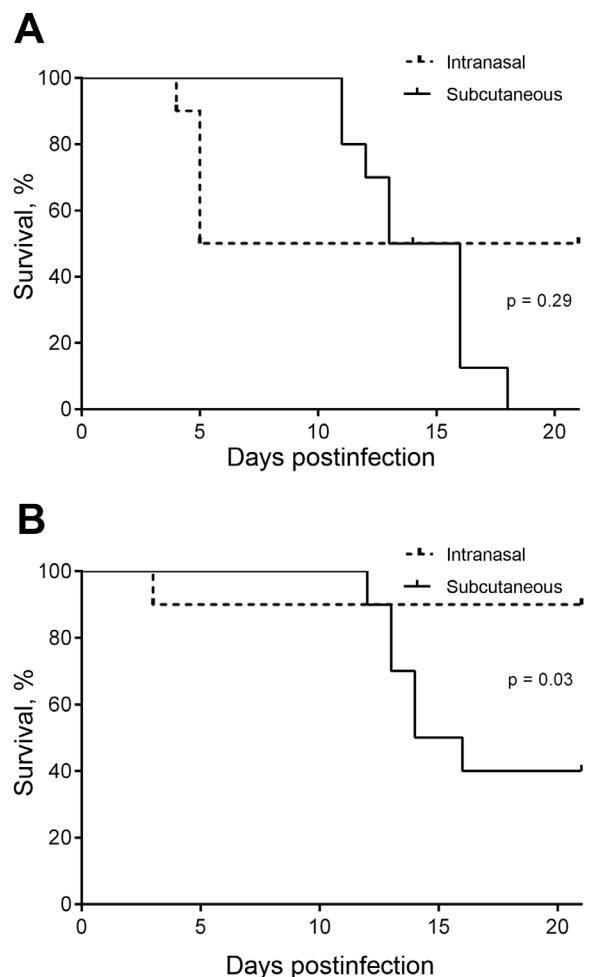


Figure 1. Comparison of survival after intranasal and subcutaneous infection of BALB/c mice with equivalent doses of the neurologic *Burkholderia pseudomallei* isolates MSHR435 (5×10^2 CFU) (A) and MSHR1153 (4.5×10^2 CFU) (B), Northern Territory, Australia, October 1989–October 2012. This inoculation dose was $>50\times$ the 50% infectious dose for MSHR435 and MSHR1153, delivered by intranasal or subcutaneous inoculation. Data are expressed as percentage survival; 10 mice were monitored within each group for 21 days postinfection.

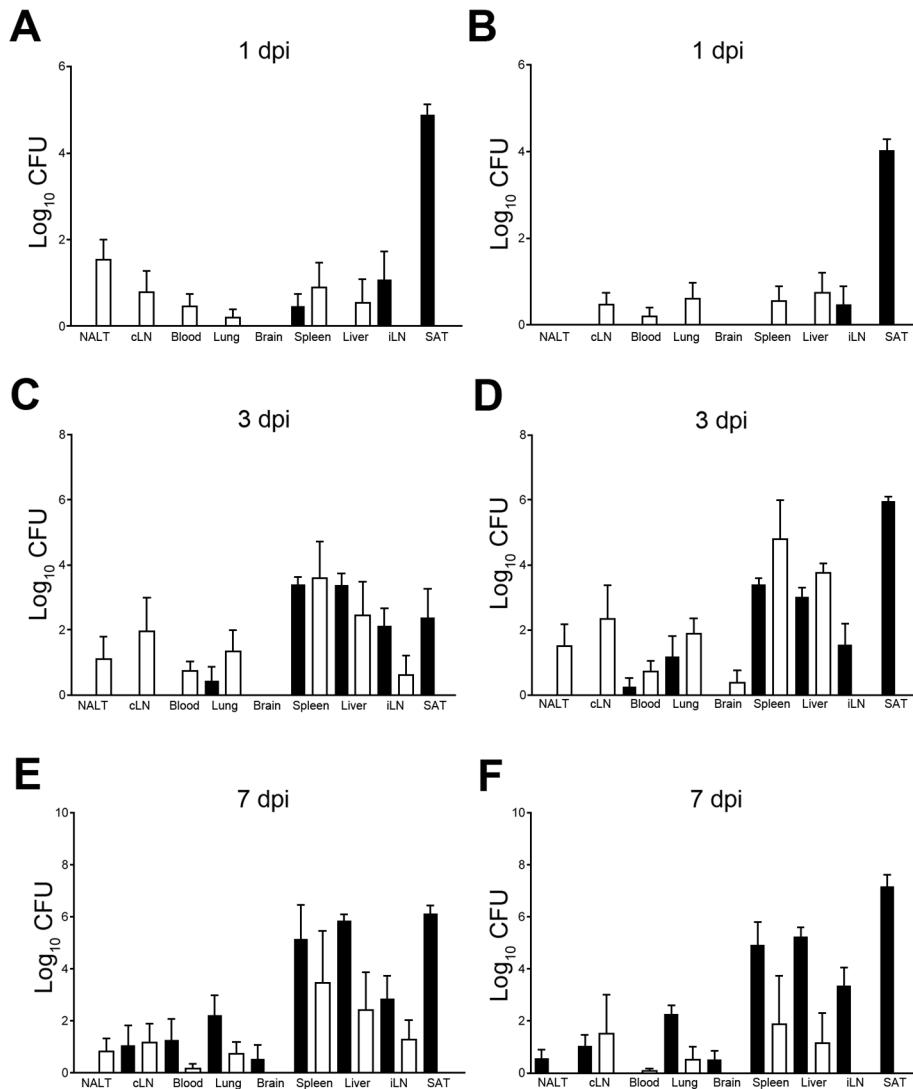


Figure 2. Comparison of *Burkholderia pseudomallei* loads in organs of BALB/c mice at days 1 (A, B), 3 (C, D) and 7 (E, F) after intranasal (white bars) and subcutaneous (black bars) infection with the neurologic isolates MSHR435 (5×10^2 CFU; panels A, C, E) and MSHR1153 (4.5×10^2 CFU; panels B, D, F), Northern Territory, Australia, October 1989–October 2012. Bacterial loads were assessed in NALT, cLN, iLN, blood, lung, brain, spleen, liver, and SAT at the indicated dpi. Five mice were assessed at each time point. Data are expressed as mean Log₁₀ CFU per tissue \pm SEM. cLN, cervical lymph nodes; dpi, days postinfection; iLN, inguinal lymph nodes; NALT, nasal-associated lymphoid tissue; SAT, subcutaneous adipose tissue. Error bars indicate standard error of the mean.

infection, consistent with the tendency for neurologic signs to develop after 8 dpi. The levels of bacteria recovered from brain after intranasal or subcutaneous infection with MSHR435 or MSHR1153 did not differ between 3 dpi and 7 dpi.

Discussion

Neurologic melioidosis is a serious, potentially fatal form of *B. pseudomallei* infection (5–7,16). The increased incidence of neurologic melioidosis in persons without recognized risk factors emphasizes the potential public health threat from this form of the disease. The contribution of bacteria- and host-specific factors in the pathogenesis of neurologic melioidosis is poorly understood, as are the potential roles of different modes of infection, such as percutaneous versus respiratory inoculation. Our study evaluated whether *B. pseudomallei*

strains isolated from patients with neurologic melioidosis showed higher virulence in animal models of melioidosis than did strains isolated from patients with nonneurologic melioidosis. We found a trend for higher virulence for neurologic isolates than for nonneurologic isolates in an animal model, regardless of route of infection. However, neurotropism was not a unique characteristic of isolates from patients with neurologic melioidosis.

Consistent with the spectrum of clinical presentations of melioidosis, dissemination of *B. pseudomallei* in mice varied substantially. Neurologic signs developed in BALB/c and C57BL/6 mice 8–12 dpi. CNS involvement was not unique to neurologic isolates; we also observed head tilt and limb paralysis in mice infected with nonneurologic isolates. Although neurologic involvement was more commonly associated with intranasal inoculation, signs of CNS infection also occurred after subcutaneous infection.

In most instances, subcutaneous infection resulted in localized abscesses in joints of hind limbs and vertebral column, causing hind leg paresis. However, neurologic signs occasionally developed in the absence of lesions at the subcutaneous injection site. When we compared intranasal and subcutaneous infection, we found a similar pattern of dissemination of neurologic isolates, which suggests that no predilection exists for neurologic isolates to invade by the respiratory route.

Our study provides evidence that *B. pseudomallei* isolates from patients with neurologic melioidosis do not demonstrate selective neurotropism in an experimental model. Rather, a subset of *B. pseudomallei* isolates appear to have unique virulence factors that facilitate rapid dissemination to multiple tissues, including the CNS, after both intranasal and subcutaneous exposure. We propose that this group of isolates is associated with severe disease progression and increased rates of death. This finding has valuable public health ramifications for initiating appropriate and timely therapy after exposure to systemically invasive *B. pseudomallei* strains. Studies focused on identifying virulence factors of *B. pseudomallei* that influence systemic spread, together with an improved understanding of the host–pathogen interactions that influence the progression to different forms of melioidosis, will be instrumental in identifying and evaluating future vaccine candidates and novel therapeutics for this potentially life-threatening disease.

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Dr. Morris is a postdoctoral researcher in the Australian Institute of Tropical Health and Medicine. Her research interests include the immunopathogenesis of *B. pseudomallei* infection.

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Molecular Epidemiology and Genetic Diversity of *Orientia tsutsugamushi* from Patients with Scrub Typhus in 3 Regions of India

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Scrub typhus, an acute febrile illness that is widespread in the Asia-Pacific region, is caused by the bacterium *Orientia tsutsugamushi*, which displays high levels of antigenic variation. We conducted an investigation to identify the circulating genotypes of *O. tsutsugamushi* in 3 scrub typhus–endemic geographic regions of India: South India, Northern India, and Northeast India. Eschar samples collected during September 2010–August 2012 from patients with scrub typhus were subjected to 56-kDa type-specific PCR and sequencing to identify their genotypes. Kato-like strains predominated (61.5%), especially in the South and Northeast, followed by Karp-like strains (27.7%) and Gilliam and Ikeda strains (2.3% each). Neimeng-65 genotype strains were also observed in the Northeast. Clarifying the genotypic diversity of *O. tsutsugamushi* in India enhances knowledge of the regional diversity among circulating strains and provides potential resources for future region-specific diagnostic studies and vaccine development.

Scrub typhus is a vector-borne, acute febrile illness caused by *Orientia tsutsugamushi*, an obligate intracellular, gram-negative bacterium. Scrub typhus is widespread in the Asia-Pacific region, known as the “tsutsugamushi triangle.” Mite larvae, or chiggers, of the genus *Leptotrombidium* transmit the causative bacteria to humans through their bite. The infection is maintained in nature through transovarial transmission in the vector and a reservoir in small mammals (1,2). Clinical signs and symptoms of scrub typhus in humans are largely nonspecific, and if infection is not treated promptly and appropriately, it carries a high mortality rate (3).

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The *Orientia* genome has a high degree of plasticity and is considered to be the most highly repetitive bacterial genome sequenced (4). This diversity is a result of high numbers of intragenomic deletions, duplications, and rearrangements with transposable and conjugative elements. These recombinations and rearrangements are unlikely to occur in dead-end hosts, but the details of this process are unclear (5).

Clarifying the epidemiology and genetic diversity of *O. tsutsugamushi* strains is essential to the development of rapid diagnostics and vaccines in disease-endemic areas. These efforts would also help in the early recognition and treatment of the disease. Currently, the most widely used method for strain classification is sequence analysis of the 56-kDa type-specific antigen (TSA), an immunodominant outer membrane protein unique to *O. tsutsugamushi*. With an open reading frame (ORF) of ≈1,600 bp, the 56-kDa TSA contains 516–541 amino acids and is involved in host cell invasion through the binding of fibronectin (6). Four hypervariable domains in this region, variable domains (VD) I–IV, are responsible for the large degree of antigenic variation in this gene. The direct interaction with the host, uniqueness to *O. tsutsugamushi*, and high level of variability make this protein an attractive target for studying the genetic variation among strains. This region is also highly immunogenic, making it a potential candidate as a vaccine target.

The process of conventional serotyping was a complex procedure, requiring reference serum samples and antigens, and is of limited use today. Greater diversity among the strains has been revealed by using molecular genotyping methods. Antigenic variations in *O. tsutsugamushi* from patients and rodents in different scrub typhus–endemic regions have been reported by testing using the 56-kDa TSA, which has led to identification of several new subtypes (1), such as Japanese Gilliam, Japanese Karp, Kawasaki, Kuroki, and Shimokoshi, in addition to the previously described prototypes Karp, Kato, and Gilliam (7,8).

Given the broad endemicity of scrub typhus in the Asia-Pacific region and variations in clinical manifestations that may be attributable to strain variation, thorough investigation into the regional distribution of genotypes is warranted. This study was conducted to identify the circulating 56-kDa antigen genotypes in 3 scrub typhus-endemic geographic regions of India: South India, Northern India, and Northeast India.

Methods

Study Population

All patients who sought care for fever and suspected scrub typhus at the 3 recruiting centers (Christian Medical College in Vellore, Indira Gandhi Medical College in Shimla, and the Dr. H. Gordon Roberts Hospital in Shillong) during September 2010–August 2012 were evaluated. A detailed clinical assessment of each patient for the signs and symptoms of scrub typhus, including a careful search for an eschar and basic laboratory studies, was documented by using a standardized form. Additional investigations, including blood cultures, quantitative buffy coat (testing for malarial parasites), abdominal ultrasound, and serologic testing for the causative agents of leptospirosis and dengue, were performed to rule out other common infections. Detection of *O. tsutsugamushi* was performed by using the Scrub Typhus Detect IgM ELISA (InBios International, Inc., Seattle, WA, USA) according to the manufacturer's instructions; an optical density (OD) >0.5 was considered positive. De-roofed eschar samples were collected and stored in absolute alcohol at -70°C until DNA extraction. Patients with clinical illness compatible with scrub typhus, including an eschar and positive results for serum samples testing by IgM ELISA, were included in the study. The study was approved by the Institutional Review Board and Ethics Committee of Christian Medical College, and informed consent was obtained from all patients.

DNA Amplification and Sequence Analysis

DNA was extracted from the homogenized eschar samples by using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. A standard PCR, targeting the 56-kDa protein, was performed as described by using the primers OtsuF: 5'-AATTGCTAGTGCAATGTCTG-3' and OtsuR: 5'-GGCATTATAGTAGGCTGAG-3' (Sigma Aldrich, Bangalore, India) (9). This region encompasses ≈ 410 bp and contains the VD I–III hypervariable regions. The PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN) according to the manufacturer's instructions; products were then subjected to a sequencing reaction using the BigDye Terminator Mix (Applied Biosystems, Foster City, CA, USA) and subsequent automatic

sequencing using the ABI 310 Genetic Analyzer (Applied Biosystems). PCR and sequencing reactions were carried out in the Infectious Diseases Research laboratories at Christian Medical College in Vellore.

The sequences obtained were identified by comparison with sequences available in GenBank by using BLAST (<http://blast.ncbi.nlm.nih.gov>). All sequences obtained were submitted to GenBank (accession nos. KC153061–KC153085 and KF777306–KF777328 for samples from Vellore; KF777265–KF777290, KF777292, KF777294–KF777305 for samples from Shimla; and KF777329–KF777357 for samples from Shillong). Phylogenetic and molecular evolutionary analyses were conducted by using MEGA5 (10). The study sequences and reference sequences downloaded from GenBank were aligned by using ClustalW (<http://www.clustal.org>) and trimmed to the appropriate size. A phylogenetic tree with 1,000 bootstrap replications was constructed by using neighbor-joining methods with distances calculated by the maximum composite likelihood. Statistical analysis was performed by using SPSS software version 16.0 for Windows (SPSS IBM, Armonk, NY, USA). Descriptive data are given as mean (SD) or median (range).

Results

A total of 263 eschar samples were obtained from patients with confirmed scrub typhus from the 3 study sites: 95 from Vellore in South India, 72 from Shimla in Northern India, and 96 from Shillong in Northeast India. Of the patients from whom these samples were collected, 115 (43.7%) were male and 148 (56.3%) female. Mean patient age was 40 ± 12 years.

Of the 263 samples, 209 (79.5%) were successfully amplified by using 56-kDa conventional PCR. Of the 209 amplified samples, 130 (62.2%) yielded good sequencing reads (58 from Vellore, 42 from Shimla, and 30 from Shillong). These reads were then used for further data analysis.

Sequence Analysis

Sequence analysis revealed that Kato-like strains predominated; 80 (61.5%) of the 130 total samples analyzed yielded Kato-like strains, followed by Karp-like strains with 36 (27.7%) samples and Gilliam and Ikeda strains with 3 (2.3%) samples each. Four (3.1%) samples from Shillong were similar to Neimeng-65, and 4 (3.1%) from Shimla were similar to IHS-II. The Karp-like amplicons showed a nucleotide similarity of 88% to 95% to the Karp reference strain (GenBank accession no. AY956315), and the nucleotide similarity for Kato-like strains to the US Centers for Disease Control and Prevention (CDC) Kato reference strain (GenBank accession no. AY836148) ranged from 90% to 94%. Most sequences showed 90%–96% similarity to Hualien strains from Taiwan.

The distributions of genotypes by center are displayed in the Table. Among the 58 samples analyzed from Vellore, 45 (77.5%) were Kato-like, and most of those (35, 77.7%) showed 92%–96% sequence similarity to the Hualien-3 strain from Taiwan. Two Kato-like amplicons were 94% similar to another strain from Taiwan (GenBank accession no. KM0607b), and 4 others were 92%–95% similar to strain TA678 from South Korea. The remaining showed similarities ranging from 92% to 95% to various strains such as MZ02, KM06, and HL03 from Taiwan. Eleven (18.9%) samples from Vellore yielded Karp-like amplicons, and these showed 95%–98% sequence similarities to the clone T0122244_KH from Cambodia and Vietnam. One Gilliam strain found in Vellore showed 99% similarity to the BA344_1 Gilliam strain from Thailand (GenBank accession no. JN587265). One Ikeda strain was also found in Vellore. Phylogenetic analysis of the Vellore strains with reference strains showed 2 distinct clusters (Figure 1, panel A); one of the clusters included 11 strains from Vellore along with the Karp reference strains Taiwan CDC Karp, Kp-1, and Kp-2a.

Kato-like sequences also predominated among the 30 analyzed sequences from Shillong; 17 (56.6%) samples belonged to this strain. These genotypes, however, were generally most similar (94%–95%) to the Hualien-13 strain from Taiwan. Some were also highly similar (98%) to the clone T1116018_KH from Cambodia and Vietnam, and 1 was 99% similar to the reference strain Taiwan CDC Kato. Seven samples (23.3%) from Shillong were Karp-like strains, 2 (6.6%) were Gilliam strains, and 4 (13.3%) were Neimang-65 strains. Two major clusters were observed on phylogenetic analysis, with a few of the strains clustering with the Hualien-13 strains and the remaining strains scattered in a second, larger cluster with several subgroups (Figure 1, panel B).

Of the 42 sequences analyzed from Shimla, 18 (42.8%) were Kato-like, and most showed 94%–96% similarity to the Hualien-3 strain. The samples from Shimla also included 18 (42.8%) Karp-like samples, which were most similar to the MZ01 strain obtained from chiggers from Taiwan. Two (4.7%) samples were found to be of the Ikeda strain and 4 (9.5%) of the IHS-II strain. Two distinct clusters with subgroups were observed on phylogenetic analysis (Figure 1, panel C).

A comparison of sequences obtained in this study revealed that the samples from the 3 centers clustered separately on the neighbor-joining tree. The Vellore strains formed a distant clade, whereas the Shillong and Shimla strains clustered more closely together (Figure 2, <http://wwwnc.cdc.gov/EID/article/21/1/14-0580-F2.htm>).

Discussion

Scrub typhus has been recognized as a major cause of undifferentiated acute febrile illness in India (11). Previous studies have reported the prevalence of scrub typhus in Vellore and Shimla (12–15), whereas reports from Shillong are few and fairly recent (16,17). Scrub typhus has been reported from states throughout India: Kerala, Karnataka, Andhra Pradesh, and Tamil Nadu in South India; Bihar, Maharashtra, Jammu Kashmir, Himachal Pradesh, Uttaranchal, and Rajasthan in Northern India; and Meghalaya, Sikkim, and West Bengal in Northeast India (9,12,18–24). Despite the broad effect of scrub typhus in India, little genotyping has been performed, with results available only from Himachal Pradesh (9). Similarly, although scrub typhus has been reported from neighboring countries, such as Pakistan, Myanmar, and Nepal, genotype data are lacking from these regions, making comparisons difficult (25,26).

In this study, we identified the circulating genotypes of *O. tsutsugamushi* in 3 scrub typhus–endemic regions in India by analyzing a variable portion of the 56-kDa antigen gene. Overall, 80 (61.5%) of 130 samples were Kato-like strains, which have previously been found by serologic testing and genotyping in other locations, primarily Japan and Cambodia, but not as a predominant strain (1,4,27,28). The strains we identified were largely similar to the Hualien-3 reference sample, which was originally reported from Taiwan (6). However, some Kato-like strains showed closer similarity to the TA678 strain from South Korea. In Shillong, the Kato-like strains were most similar to another Taiwan strain, Hualien-13. Other Kato-like strains that bore a closer resemblance to the clone T1116018_KH from Cambodia and Vietnam were also found in Shillong.

The highest proportion of Kato-like strains (77.5%) was found at Vellore in South India, with progressively smaller proportions of this strain seen as locations progressed to the north. The highest proportion of Kato-like strains in a single region (21.5%) was previously reported from Cambodia (4); South India's climate is more similar to Cambodia's climate than to Northern India's climate, so temperature or other environmental factors may play a role in the selecting for this strain. However, most of the Kato-like strains we found, particularly from Vellore, resembled strains from Taiwan rather than Cambodia, which could argue against a climate-based selection process. Kato-like strains in Shillong, which is the geographically closest of our sites to Cambodia, resemble the strains found in Southeast Asia.

Table. Geographic distribution of *Orientia tsutsugamushi* genotypes in 3 regions of India, September 2010–August 2012

Genotype	Vellore, n = 58	Shimla, n = 42	Shillong, n = 30	Total, n = 130
Kato-like	45 (77.5)	18 (42.8)	17 (56.6)	80
Karp-like	11 (18.9)	18 (42.8)	7 (23.3)	36
Gilliam	1 (1.7)	0	2 (6.6)	3
Ikeda	1 (1.7)	2 (4.7)	0	3
IHS-II	0	4 (9.5)	0	4

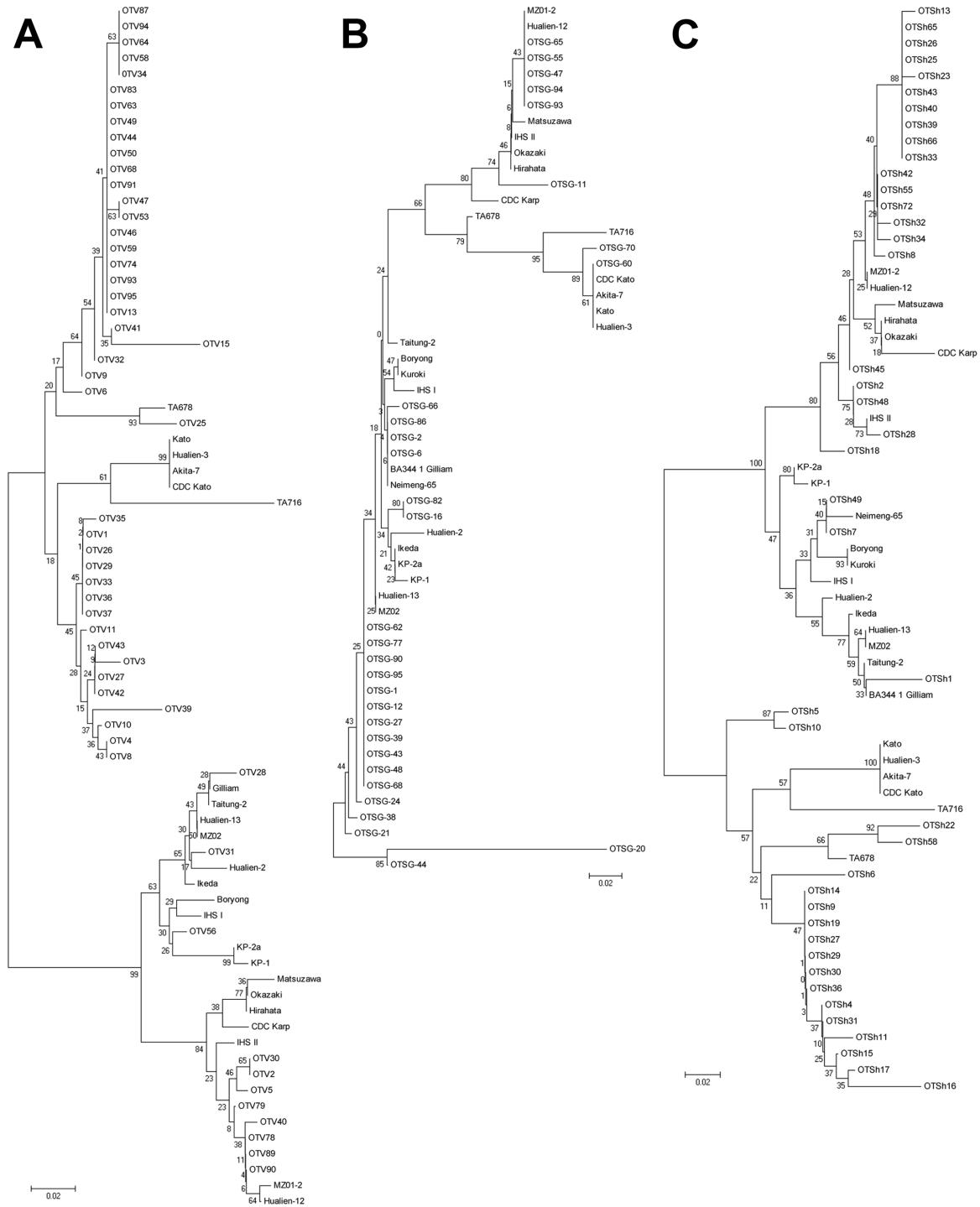


Figure 1. Phylogenetic distribution of *Orientia tsutsugamushi* isolates from scrub typhus patients in Vellore (A), Shillong (B), and Shimla (C), India, September 2010–August 2012. Isolates were identified on the basis of the 56-kDa TSA gene. Evolutionary history was inferred by using the neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed by using the maximum composite likelihood method (10). Scale bars indicate base substitutions per site. Sequences identified in this study were deposited in GenBank under accession nos. KC153061–KC153085 and KF777306–KF777328 (OTV), KF777329–KF777357 (OTSG), and KF777265–KF777290, KF777292, and KF777294–KF777305 (OTSh). OTV, *O. tsutsugamushi* from Vellore; OTSG, *O. tsutsugamushi* from Shillong; OTSh, *O. tsutsugamushi* from Shimla.

Karp-like strains, although less common than Kato-like in our study, are more widespread globally, reported by serologic testing and genotyping in Southeast Asia (Thailand, Vietnam, Cambodia, the Philippines, and Malaysia), Japan, southern China, Taiwan, Mongolia, Oceania, and Australia; these strains also are cited as the most common circulating strain in several Southeast Asia countries and in Japan (1,3,27–30). By serologic testing, these strains have been previously reported in India and in Pakistan just to the north (1). In our study, Karp-like strains were at their highest proportion at Shimla in the northern part of the country (equal to the amount of Kato-like strains), with progressively smaller proportions of this grouping successively farther south. Most Karp-like strains from Vellore showed high sequence similarity to strains previously found in Cambodia and Vietnam; strains in 5 samples showed a high degree of similarity to the Ld-1a strain (GenBank accession no. JN415541), which was reported from trombiculid mites in Thailand. Unfortunately, sequencing data were not sufficient to assess for similarities in the Karp-like samples from Shillong or Shimla.

Our study also identified Gilliam-like strains in Vellore and Shillong; all of these sample strains were similar to the BA244_1 strain previously found in mites from Thailand. Gilliam strains have been reported in a variety of locations, including Japan, Korea, China, Taiwan, Thailand, Russia, Tajikistan, and Northern India (1,29,31–35). All of our samples, including those from Shillong in Northeast India, were most similar to genotypes previously seen in Thailand, not to the Gilliam-like strain from Myanmar (GenBank accession no. DQ286233) that was previously reported in Northern India. In addition, we isolated Neimeng-65 strains in Shillong. This finding is not surprising in Northeastern India, considering the proximity of the area to the border of China, where Neimeng-65 was originally reported in rodents from Inner Mongolia and Xinjian (GenBank accession no. DQ514319). We also found Ikeda-like strains from Vellore and Shimla; these strains were previously reported primarily from Japan (27).

Initial molecular descriptions of *O. tsutsugamushi* in India were reported in Shimla, where 2 new genotypes, IHS-I (GenBank accession no. DQ286233DQ530440) and IHS-II (GenBank accession no. DQ286233DQ530441), were identified based on partial 56k-Da TSA sequences (9). Strains in 4 samples, all from Shimla, in this study showed the highest nucleotide similarity to the IHS-II strain. Seerangayee strains and the Kuroki strain of the Boryong cluster, previously reported in India (1,34), were not identified in our study. Other groupings, such as Kawasaki, TA673, TA716, and Japanese Gilliam, were also not observed.

Our study has limitations. Not all scrub typhus patients have an eschar, so we could not run PCR testing on samples from all patients. If some strains are more likely than others to produce an eschar, some strains may have been over-

underrepresented in our study. In addition, not all eschar samples were amenable to good sequencing, which could also have skewed our results.

In summary, we identified the circulating genotypes of *O. tsutsugamushi* in 3 scrub typhus–endemic regions of India. Kato-like strains were found to be predominant in the South and Northeast, whereas an equal prevalence of Karp-like and Kato-like strains was observed in Northern India. The prevalence of antigenic diversity can have wider implications in vaccine development; also, a potential association between strain variation and pathogenicity of scrub typhus has reported in mice studies (36,37). In addition, targeted serodiagnosis will require further knowledge of this variability. Thus, an accurate picture of the local antigenic diversity will be essential for the development of region-specific vaccines and diagnostics.

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Rates and Risk Factors for Coccidioidomycosis among Prison Inmates, California, USA, 2011¹

Charlotte Wheeler, Kimberley D. Lucas, and Janet C. Mohle-Boetani

Coccidioidomycosis is a disease acquired by inhaling spores of *Coccidioides immitis*, a fungus found in certain arid regions, including the San Joaquin Valley, California, USA, where 8 state prisons are located. During 2011, we reviewed coccidioidomycosis rates at 2 of the prisons that consistently report >80% of California's cases among inmates and determined inmate risk factors for primary, severe, and disseminated coccidioidomycosis. Inmates of African American race/ethnicity who were ≥ 40 years of age were at significantly higher risk for primary coccidioidomycosis than their white counterparts (odds ratio 2.0, 95% CI 1.5–2.8). Diabetes was a risk factor for severe pulmonary coccidioidomycosis, and being African American was a risk factor for disseminated disease. These findings contributed to a court decision mandating exclusion of African American inmates and inmates with diabetes from the 2 California prisons with the highest rates of coccidioidomycosis.

Coccidioidomycosis, commonly called “cocci” or “valley fever,” is an illness caused by *Coccidioides immitis* and *C. posadasii*, soil-dwelling fungi found in certain arid regions of the southwestern United States, northern Mexico, and Central and South America. Infection is acquired by inhaling airborne fungal spores and is not spread person-to-person. Sixty percent of *Coccidioides* infections are asymptomatic, and most symptomatic infections consist of self-limited, flu-like illnesses. A small proportion of cases result in prolonged illness that may require lifelong treatment and can be life-threatening, particularly the 3%–5% in which the disease disseminates outside of the lungs. Infection, except in very rare cases, confers lifelong immunity.

In 2005, the medical executive team of the California Department of Corrections and Rehabilitation (CDCR) informed the California Department of Public Health (CDPH) that physicians at 2 prisons for adult men (prison

X and prison Y) reported an increase in the number of inmates with coccidioidomycosis. The prisons are located <15 miles apart from one another in a *Coccidioides*-endemic area of California's San Joaquin Valley. In response to the call, CDPH investigated the cases at prison X and confirmed rates of disease >400 \times higher than those of the surrounding county. Additionally, CDPH performed a cohort study at prison X and identified an increased risk for coccidioidomycosis among African American inmates, inmates ≥ 40 years of age, and inmates who resided on a particular yard (J. Yuan, unpub. data).

In 2006, CDPH made recommendations concerning coccidioidomycosis. In response, the California Correctional Health Care Services (CCHCS) (the medical arm for California inmates) instituted policies for educating inmates and staff about coccidioidomycosis and for excluding inmates with immunocompromising conditions or severe chronic obstructive pulmonary disease from California prisons in 3 coccidioidomycosis-endemic counties. In addition, the agency mandated the cancellation of planned construction to expand prison X. During subsequent years, prisons X and Y took measures to control ambient dust (and presumably spores) by planting native grasses and shrubs on bare grounds. In December 2011, prison X applied a soil-stabilizing emulsion to most of the grounds within the prison's perimeter. Despite these efforts, high coccidioidomycosis attack rates continued to be reported from these institutions (CCHCS coccidioidomycosis surveillance system, unpub. data).

The purpose of this study was to review rates of coccidioidomycosis at prisons X and Y, to reevaluate the population for risk factors for development of primary disease, as well as to evaluate inmate risk factors for development of the most debilitating forms of coccidioidomycosis. We used the study results to improve the policies and practices for protecting California inmates from coccidioidomycosis and its most serious sequelae.

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Materials and Methods

Coccidioidomycosis Incidence and Cases per Person-Years

We calculated coccidioidomycosis incidence in 2 ways: 1) as a proportion of the population at risk, and 2) as the number of cases per person-years. Because community coccidioidomycosis rates are measured by incidence proportions, we calculated inmate rates by the same measurement to enhance comparison. Because cases per person-years is the recommended measure of disease incidence in a dynamic population, and because inmates are frequently moved from one prison to another throughout a year, calculating inmate cases per person-years gave us a measure against which to check coccidioidomycosis incidence proportions. Our concern was that the coccidioidomycosis incidence proportions might overestimate coccidioidomycosis rates in this study population.

To calculate coccidioidomycosis incidence proportion in the prisons, we derived coccidioidomycosis case counts from a surveillance system implemented in California prisons in 2007. Public health nurses assigned to CDCR prisons report coccidioidomycosis cases to the CCHCS Public Health Branch. Cases must meet the National Notifiable Diseases Surveillance System case definition for coccidioidomycosis (1). We calculated the yearly rates in prisons by dividing the surveillance-derived case counts by the published mid-year inmate populations (2). We obtained city coccidioidomycosis counts from local county health departments (F. Aranki, M. MacLean, unpub. data), and county and state coccidioidomycosis counts from data published by CDPH (3). We calculated annual community rates by dividing coccidioidomycosis counts by mid-year population estimates obtained from local health departments (for cities) and from the California Department of Finance (for counties and the state of California) (4,5). Because community data contain prison counts, prisons X and Y coccidioidomycosis counts were subtracted from their respective community coccidioidomycosis counts (city, county, and state counts), and prisons X and Y population counts were subtracted from their respective community populations. We compared prisons X and Y coccidioidomycosis incidence proportions to the incidence proportions of their surrounding communities and to those of Kern County and the state of California. Kern County coccidioidomycosis incidence proportions are benchmarks because Kern County consistently reports the highest coccidioidomycosis incidence of any county in California.

We calculated cases per person-years for prisons X and Y based on data from a cohort of inmates who had spent ≥ 1 night in 2011 at either prison X or Y (study cohort). This cohort was subject to certain exclusions: inmates who spent time at both institutions during 2011, inmates who received a coccidioidomycosis diagnosis before 2011, and inmates who received a coccidioidomycosis diagnosis in 2011 at

a prison other than X or Y. To derive the total number of person-years spent at the prisons, we summed the number of days each inmate was incarcerated at prison X or Y and divided that sum by 365.

Risks for Primary Coccidioidomycosis

We defined primary coccidioidomycosis as an illness compatible with coccidioidomycosis that caused an inmate to seek medical attention and that was confirmed as coccidioidomycosis by a laboratory test. We collected primary cases from the CCHCS coccidioidomycosis surveillance system. We performed a cohort study to determine risk factors for primary coccidioidomycosis based on race/ethnicity, age, and whether the inmate had diabetes mellitus (DM); the latter was included in the model because studies and case series have identified an association between DM and complications of coccidioidomycosis (6–9). For this analysis, we again used the study cohort dataset that we had used to determine the coccidioidomycosis cases per person-years. Race/ethnicity, birthdate, and DM status were available for each inmate in the study cohort. Race/ethnicity is recorded on an inmate's arrival into the CDCR prison system and is chosen by the inmate from a list of 27 race/ethnicities that includes a category called "other."

We grouped the race/ethnicity values into 5 categories: African-American (for those inmates who identified themselves as black or Jamaican); Asian/Pacific Islander (for those who identified themselves as Cambodian, Chinese, Filipino, Guamanian, Hawaiian, Japanese, Korean, Laotian, Other Asian, Pacific Islander, Samoan, Thai, or Vietnamese); Hispanic (for those who identified themselves as Colombian, Cuban, Guatemalan, Hispanic, Mexican, Nicaraguan, Puerto Rican, or Salvadoran); other (for those who identified themselves as American Indian, Indian, or other); and white (for those who identified themselves as white).

We calculated age at midyear 2011 and included age as a continuous variable in our model. DM status of each inmate was determined on the basis of laboratory (hemoglobin A1C results) and pharmacy information (diabetic medication prescriptions). To determine risks for primary coccidioidomycosis, we explored interaction by using stratified analyses and then performed logistic regression on a model that contained all variables and interaction terms.

Risks for Severe and Disseminated Coccidioidomycosis

To determine risk factors for severe and disseminated disease, we performed a case-control study. We defined severe disease as a case of coccidioidomycosis that was confined to the lungs (nondisseminated), for which the patient required ≥ 10 days of hospitalization. A patient was determined to have severe coccidioidomycosis if he was in the hospital for ≥ 10 days during which all hospital discharge International Classification of Disease, Ninth Revision

(ICD-9), codes indicated nondisseminated coccidioidomycosis (ICD-9, codes 114.0 or 114.4–114.9). We defined disseminated coccidioidomycosis as disease in which the patient had a discharge ICD-9 code for disseminated coccidioidomycosis (ICD-9 codes of 114.1–114.3) for any hospitalization. Cases were derived from the CCHCS hospitalization discharge dataset for the period July 1, 2010, through April 11, 2013. This dataset contained hospitalization data for CDCR inmates incarcerated in any of California's 33 adult prisons. Controls were patients in whom coccidioidomycosis was diagnosed in 2011 at prison X or Y who had not been hospitalized as of April 11, 2013. We evaluated the variables of race/ethnicity, age, and DM status by using logistic regression to predict severe and disseminated coccidioidomycosis. For the models for severe and disseminated coccidioidomycosis, our numbers were not robust enough to support models with interaction terms.

Statistical Analyses

We used SAS 9.2 (SAS Institute; Cary, NC, USA) for all statistical analyses. The *p* value for statistical significance was set at ≤ 0.05 .

Results

Coccidioidomycosis Incidence and Cases per Person-Years

For 2011, the coccidioidomycosis cases per 100,000 population for prisons X and Y were 6,934 and 3,799, respectively, 1–2 orders of magnitude higher than the rates at the other 6 prisons in 3 counties to which coccidioidomycosis was endemic (Table 1). These rates were also

Table 1. Coccidioidomycosis cases in prison X, prison Y, and prisons 1–6; in the communities surrounding the prisons X and Y and in Kern County, California; and in the state of California, USA, 2011

Location	No. cases	Mid-year population	Cases/100,000 population
Prison X	317	4,572	6,934
Prison Y	218	5,738	3,799
Prison 1	3	5,647	53
Prison 2	11	6,389	172
Prison 3	10	5,051	198
Prison 4	10	4,682	214
Prison 5	11	4,938	223
Prison 6	14	5,908	237
Communities			
City of prison X*	172	12,821	1,342
City of prison Y†	53	9,210	575
County of prison X	376	934,875	40
County of prison Y†	131	145,961	90
Kern County	2,568	848,958	302
California*†	4,607	37,559,818	12

*Prison X case and population counts were subtracted from these surrounding communities' case and population counts.

†Prison Y case and population counts were subtracted from these surrounding communities' case and population counts.

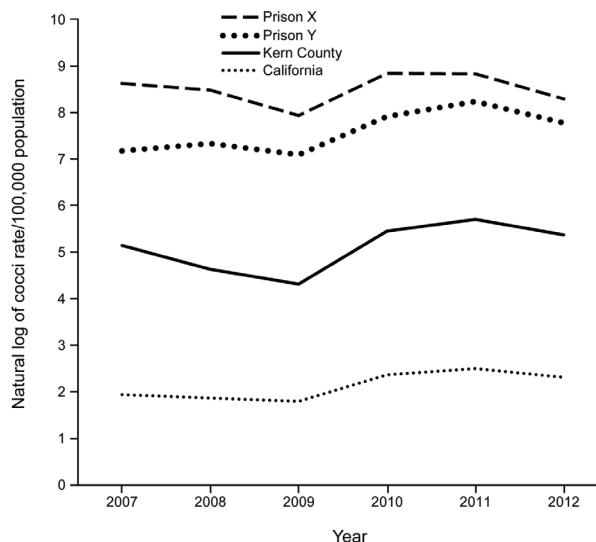


Figure. Natural log of coccidioidomycosis cases per 100,000 population for prison X, prison Y, Kern County, and the state of California, 2007–2012.

an order of magnitude higher than the 2011 rate for Kern County, and that difference was consistent over the period 2007–2012 (Figure).

In 2011, 16,560 inmates spent at ≥ 1 night in prison X or prison Y. Of these, 834 were excluded from the analysis because they had been given a diagnosis of coccidioidomycosis before 2011 or at another institution in 2011; 155 were excluded because they were incarcerated at both institutions during 2011. Of the remaining 15,571 inmates, 6,064 inmates had been incarcerated at prison X and 9,507 at prison Y. In all, 516 had a diagnosis of coccidioidomycosis in 2011, 304 from prison X and 212 from prison Y. The 6,064 inmates of prison X spent 4,037 person-years at the prison, and the 9,507 inmates of prison Y spent 5,464 person-years at the prison. The coccidioidomycosis cases per 100,000 person-years was 7,530 for prison X and 3,880 for prison Y.

Risks for Primary Coccidioidomycosis

Of the 15,571 inmates in the cohort, 6,558 (42%) were Hispanic, 4,380 (28%) were white, and 3,728 (24%) were African American. Asian/Pacific Islanders numbered 128 (1%), of which 36 were identified as Filipino. The remaining 777 (5%) inmates were categorized as other; of these, 183 (1% of the total cohort) were identified as American Indian. The median age of the inmates in the study cohort was 39 years (range 17–89 years; 10th percentile 24 years, 90th percentile 56 years).

In a univariable model, age was significantly associated with primary coccidioidomycosis (OR 1.009, 95% CI 1.002–1.016). Stratified analyses suggested an interaction between age and race/ethnicity in predicting primary

Table 2. Association of primary coccidioidomycosis with prison of incarceration, diabetes status, and the number of days incarcerated among inmates, California, USA, 2011*

Characteristic	No. (%) ill	No. (%) not ill	aOR	95% CI
Prison of incarceration				
Prison X	304 (58.9)	5,760 (38.3)	1.95	1.63–2.34
Prison Y	212 (41.1)	9,295 (61.7)	Referent	
Persons with diabetes	44 (8.5)	1,187 (7.9)	0.87	0.62–1.21
No. days at prison X or Y in 2011	NA	NA	1.007	1.006–1.009

*aOR, adjusted odds ratio; NA, not applicable; number of days incarcerated cannot be expressed as a single value for those ill and not ill.

coccidioidomycosis. We therefore created a model with variables for prison (prison X or Y), DM status, and the interaction term for age and race/ethnicity. Logistic regression on this model resulted in a significant association with primary coccidioidomycosis for incarceration at prison X (compared with incarceration at prison Y) and with days of incarceration at prison X or Y (Table 2). Also significant were African American and other race/ethnicity at ≥ 40 years of age and Hispanic race/ethnicity at ≥ 55 years of age. At age 55, African American, Hispanic, and other race/ethnicity were significantly associated with coccidioidomycosis with odds ratios of 2.5 (95% CI 1.7–3.6), 1.6 (95% CI 1.1–2.3), and 2.2 (95% CI 1.2–3.9), respectively, when compared to white persons (Table 3).

Risks for Severe and Disseminated Coccidioidomycosis

A total of 115 inmates had severe coccidioidomycosis, and 115 inmates had disseminated coccidioidomycosis (the equal numbers of severe and disseminated cases was coincidental). There were 474 prison X or Y inmates in whom coccidioidomycosis was diagnosed in 2011 who had not been hospitalized as of April 11, 2013. Logistic regression on a model containing DM status, race/ethnicity, and age resulted in a significant association between severe coccidioidomycosis and DM (OR 3.2, 95% CI 1.8–5.8) (Table 4). Logistic regression on a model containing DM status, race/ethnicity, and age resulted in a significant association between disseminated coccidioidomycosis and African American race/ethnicity (OR 1.9, 95% CI 1.1–3.4) (Table 5).

Discussion

For >5 years, 2 California prisons for adult men experienced rates of coccidioidomycosis that exceeded the rate of Kern County by 1–2 orders of magnitude. Calculations of cases per person-years for these prisons for 2011 exceeded the cases-per-midyear population figures, further confirming the high rates of coccidioidomycosis in prisons X and Y. Various theories have been proposed to explain these high rates. During its investigation, CDPH explored the possibility that a change in provider practices in 2005 (e.g., increased testing for coccidioidomycosis) might have resulted in more diagnoses of coccidioidomycosis at prison X but found that no such change had occurred (J. Yuan, unpub. data). In 2013, we explored the possible contribution of a high population turnover (and thus frequent replenishment

of susceptible persons) at prisons X and Y to the high rates, but we found no association (J. Mohle-Boetani, unpub. data). Most inmates at prisons X and prison Y resided in areas to which coccidioidomycosis was not endemic before incarceration, so their susceptibility to the disease is at least a partial explanation for high coccidioidomycosis rates compared to those of the surrounding communities. However, a naïve population does not explain the high odds for acquiring coccidioidomycosis at prison X (independent of age and race) compared to the odds at nearby prison Y. Nor does a naïve population explain the very high rates of coccidioidomycosis in the cities of prisons X and Y compared with their surrounding counties. Because coccidioidomycosis is not uniformly distributed even in the area to which coccidioidomycosis is endemic, the higher rates likely reflect either a higher concentration of ambient spores or a strain that is more pathogenic than strains found elsewhere.

Findings regarding the demographic and clinical risk factors from these analyses include the following: higher rates of primary coccidioidomycosis among persons ≥ 40 years of age are associated with certain race/ethnicities other than white; DM is associated with severe pulmonary coccidioidomycosis; and African American race/ethnicity is associated with disseminated coccidioidomycosis. These findings are not new, but have applications beyond the protection of

Table 3. Association of race/ethnicity at 3 age points with primary coccidioidomycosis among a cohort of inmates incarcerated at prison X or Y, California, USA, 2011

Characteristic	aOR*	95% CI
Race/ethnicity, age 25 y		
White	Referent	
African American	1.02	0.65–1.62
Hispanic	0.86	0.57–1.29
Asian/Pacific Islander	0.73	0.21–2.53
Other	1.26	0.65–2.44
Race/ethnicity, age 40 y		
White	Referent	
African American	1.59	1.23–2.06
Hispanic	1.18	0.92–1.51
Asian/Pacific Islander	0.93	0.33–2.58
Other	1.68	1.13–2.49
Race/ethnicity, age 55 y		
White	Referent	
African American	2.48	1.73–3.55
Hispanic	1.62	1.13–2.34
Asian/Pacific Islander	1.18	0.36–3.89
Other	2.23	1.23–3.92

*aOR, adjusted odds ratio.

Table 4. Multivariable model for the prediction of severe coccidioidomycosis in inmates with coccidioidomycosis, California, USA, 2011–2013*

Characteristic	No. (%) cases, n = 115	No. (%) controls, n = 474	aOR	95% CI
Diabetes	25 (21.7)	37 (7.8)	3.2	1.8–5.8
Race/ethnicity				
White	26 (22.6)	97 (20.4)	(ref)	
African American	41 (35.7)	152 (32.1)	0.97	0.5–1.7
Hispanic	39 (33.9)	189 (39.9)	0.80	0.5–1.4
Asian/Pacific Islander	1 (0.9)	3 (0.6)	1.47	0.1–14.9
Other	8 (7.0)	33 (7.0)	0.88	0.4–2.2
Age	NA	NA	1.001	0.983–1.019

*Cases represent patients requiring ≥ 10 days of hospitalization for nondisseminated coccidioidomycosis during July 1, 2010–April 11, 2013. Controls represent patients from prisons X and Y who received a diagnosis of coccidioidomycosis in 2011 but who had not been hospitalized as of April 11, 2013. aOR, adjusted odds ratio; NA, not applicable.

this population. The association of African American race/ethnicity and disseminated coccidioidomycosis has been reported as early as 1945 (6,10,11) and is generally accepted among researchers and clinicians in the field. However, some authors refute the existence of a predilection for primary disease by race/ethnicity (12), even though an association between African American race/ethnicity and primary coccidioidomycosis has been reported by numerous investigators (13–16). We believe our finding of this association is substantiated because it is based on the study of a population with robust numbers of persons of non-white race/ethnicity. Moreover, inmates of all races/ethnicities are similar in their activities at the institutions, such as the time they spend in the yard. Equal and prompt access to health care for all inmates is a policy of the CCHCS administration (headed by a Federal Receiver) and is monitored by outside agencies. Our other findings with regard to increased risk for coccidioidomycosis and race/ethnicity, for example, that Hispanic inmates (≥ 55 years of age) and those of other races/ethnicities (≥ 40 years of age) are at higher risk than their white counterparts, are also consistent with the literature. Gifford calculated “coccidioid granuloma” rates of Mexicans to be between those of white and African American persons (17), as is the case for our population. Other race/ethnicity, in which we included those inmates who self-reported as American Indian, and those who self-reported as other, is not clearly defined, but may represent largely mixed-race persons. That inmates were in large enough numbers in the “other” race/ethnicity category to show a significantly increased risk for primary

coccidioidomycosis compared with whites suggests the need to reevaluate the risk for mixed race individuals. Other nonwhite races, specifically Asian/Pacific Islanders, should also be investigated further, because our numbers were insufficient to assess statistically significant associations.

A limitation of our study of primary coccidioidomycosis was that many of the nonill inmates may have been previously infected and, therefore, immune to disease. Because no test for previous infection was available at the time of this study, we could not determine which inmates might have had asymptomatic infection in the past. However, this limitation would bias the findings toward the null, so does not negate our study findings. Another limitation is that inmates infected with coccidioidomycosis in their county of residence or in another prison may have had a diagnosis only after entering prison X or Y and were thus misclassified as exposed at these prisons. Although this could introduce a bias, we do not believe the acquisition of coccidioidomycosis outside of prisons X and Y would have considerable effects on our results. The numbers of cases are large for 2011, and our experience is consistent over many years that prisons X and Y report the highest coccidioidomycosis counts in our system.

On June 24, 2013, after review of the results of our analyses, and in consultation with court monitors and coccidioidomycosis experts, the United States District Court for the Northern District of California issued an order to exclude all African American inmates and inmates with DM from prisons X and Y (18). The order was enacted by CDCR.

Table 5. Multivariable model for the prediction of disseminated coccidioidomycosis in inmates with coccidioidomycosis, California, USA, 2011–2013*

Characteristic	No. (%) cases, n = 115	No. (%) controls, n = 474	aOR	95% CI
Diabetes	9 (7.8)	37 (7.8)	0.82	0.4–1.8
Race/ethnicity				
White	19 (16.5)	97 (20.4)	Referent	NA
African American	57 (49.6)	152 (32.1)	1.92	1.1–3.4
Hispanic	32 (27.8)	189 (39.9)	0.90	0.5–1.7
Asian/Pacific Islander	1 (0.9)	3 (0.6)	1.92	0.2–19.7
Other	6 (5.2)	33 (7.0)	0.94	0.3–2.5
Age	NA	NA	1.010	0.992–1.028

*Cases represent patients requiring ≥ 10 days of hospitalization for nondisseminated coccidioidomycosis during July 1, 2010–April 11, 2013. Controls represent patients from prisons X and Y who received a diagnosis of coccidioidomycosis in 2011 but who had not been hospitalized as of April 11, 2013. aOR, adjusted odds ratio; NA, not applicable.

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Clinical Course and Long-Term Outcome of Hantavirus-Associated Nephropathia Epidemica, Germany

Joerg Latus, Matthias Schwab, Evelina Tacconelli, Friedrich-Michael Pieper, Daniel Wegener, Juergen Dippon, Simon Müller, David Zakim, Stephan Seegerer, Daniel Kitterer, Martin Priwitzer, Barbara Mezger, Birgit Walter-Frank, Angela Corea, Albrecht Wiedenmann, Stefan Brockmann, Christoph Pöhlmann, M. Dominik Alscher, and Niko Braun

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Release date: December 16, 2014; Expiration date: December 16, 2015

Learning Objectives

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

- Describe clinical characteristics of acute nephropathia epidemica associated with Puumala virus hantavirus infection, based on a cross-sectional prospective survey.
- Distinguish long-term sequelae of acute nephropathia epidemica associated with Puumala virus hantavirus infection.
- Discuss serologic findings of nephropathia epidemica associated with Puumala virus hantavirus infection.

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Human infection with Puumala virus (PUUV), the most common hantavirus in Central Europe, causes nephropathia epidemica (NE), a disease characterized by acute kidney injury and thrombocytopenia. To determine the clinical phenotype of hantavirus-infected patients and their long-term outcome and humoral immunity to PUUV, we conducted a cross-sectional prospective survey of 456 patients in Germany with clinically and serologically confirmed hantavirus-associated NE during 2001–2012. Prominent clinical findings during acute NE were fever and back/limb pain, and 88% of the patients had acute kidney injury. At follow-up (7–35 mo), all patients had detectable hantavirus-specific IgG; 8.5% had persistent IgM; 25% had hematuria; 23% had hypertension (new diagnosis for 67%); and 7% had proteinuria. NE-associated hypertension and proteinuria do not appear to have long-term consequences, but NE-associated hematuria may. All patients in this study had hantavirus-specific IgG up to years after the infection.

Hantaviruses, enveloped RNA viruses of the family *Bunyaviridae*, are transmitted to humans by rodents, the natural reservoir of these viruses (1). In North America, hantavirus infection can lead to hantavirus cardiopulmonary syndrome and to case–fatality rates of up to 35% (2,3), and in Asia and Europe, infection can lead to hemorrhagic fever with renal syndrome (HFRS) (4).

In Germany, the incidence of HFRS increased from 0.09 cases/100,000 persons in 2001 to 2.47 cases/100,000 persons in 2010 (5). During October 2011–April 2012, a total of 852 HFRS cases were reported in Germany, of which 580 (68%) originated in the southern federal state of Baden-Württemberg (6). Puumala virus (PUUV), by far the most frequent cause of hantavirus disease in Germany (7), causes a milder form of HFRS (8) called nephropathia epidemica (NE). Hantavirus infections are one of the 5 most common notifiable viral diseases in Germany, along with norovirus infections, hepatitis C, influenza, and rotavirus infections (7).

NE is characterized by acute kidney injury associated with thrombocytopenia and, frequently, with proteinuria (9). Severe and often prolonged gastrointestinal symptoms

and severe back and abdominal pain also occur (10). The severity of infection with PUUV varies from subclinical disease to severe acute kidney injury, including a fatal outcome (11,12). Renal replacement therapy is required in ≈5% of hospitalized patients with acute NE (8,10,13,14), although some studies report rates of up to 25% (15,16).

Despite the high and increasing incidence of hantavirus infection, long-term follow-up data have not been reported for a large, representative cohort of patients. Moreover, it remains unclear whether NE has long-term consequences, such as hypertension, proteinuria (17), and alteration of kidney function (18,19). Two previous studies that followed up 36 Finnish patients 5 and 10 years after they experienced PUUV-associated NE, reported that the patients had increased urinary protein excretion, glomerular hyperfiltration, and elevated blood pressure at a 5-year but not a 10-year follow-up (18,19). Other reports support an association between previous hantavirus infection and subsequent hypertension (20–23).

Data on humoral immunity years after PUUV infection are not available for a representative cohort of German patients. Thus, we conducted this study in such a cohort to describe the detailed clinical phenotype of patients with clinical manifestations of PUUV infection and their long-term outcomes and humoral immunity to PUUV.

Patients and Methods

Patients

Since 2001, Germany's Protection against Infection Act (http://www.rki.de/EN/Content/Prevention/Inf_Dis_Surveillance/inf_dis_down.pdf?__blob=publicationFile, section 7) has required that confirmed cases of hantavirus infection be reported (on a named-patient basis) to local health authorities. In turn, the health authorities report HFRS cases to the Robert Koch Institute in Berlin, Germany, the central federal institution responsible for disease control and prevention.

During 2001–2012, a total of 7,476 serologically and clinically confirmed hantavirus cases were reported to the Robert Koch Institute (SurvStat, <https://www.survstat.rki.de/>) (24). In cooperation with 4 local health authorities in southern Germany (Stuttgart, Boeblingen/Sindelfingen, Esslingen, Reutlingen), we identified 1,570 (21%) of 7,476 persons who had received a clinically and serologically confirmed diagnosis of hantavirus-associated NE during 2001–2012. During September 2012–April 2013, we contacted these persons by mail, asking them to come for a follow-up examination at the outpatient clinic of Robert-Bosch-Hospital (Stuttgart). All patients gave written consent before participating in the study. The study was approved by the Ethics Committee of the Ethics Commission of the State Chamber of Medicine in Baden-Wuerttemberg (Stuttgart) (approval no. F-2012–046).

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Studies were conducted in concordance with the Declaration of Helsinki.

Data Acquisition

Acute Course of NE

Data on clinical and laboratory findings at the time of diagnosis and during the acute course of the disease were obtained from medical reports and files for each patient. Details of data acquisition are shown in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/20/1/14-0861-Techapp1.pdf>).

Follow-up

All patients (i.e., those treated as inpatients and those treated as outpatients) were included in the follow-up. Patients participated in a follow-up session once at the Robert-Bosch-Hospital outpatient clinic. Detailed past and current medical histories were obtained by using standardized case report forms, and a physical examination was conducted. The case report form, which was designed after a systematic review of the literature, included 56 questions divided into 3 sections: demographic data, time of diagnosis and acute course of the disease, and follow-up. Blood pressure for all patients was measured as recommended by the American Heart Association (25); results were classified as normotensive, prehypertension, hypertension stage 1, or hypertension stage 2, according to the classification of blood pressure for adults as described in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (26) (online Technical Appendix).

At the follow-up appointment, standard procedures were used by the Robert-Bosch-Hospital laboratory to determine complete blood counts and levels of plasma C-reactive protein, serum creatinine, urea, and second-morning urine and to perform liver function tests and blood gas analysis. Proteinuria was defined as microalbuminuria (>30 mg albumin/24 h) in urine. In addition, serum samples from all patients were analyzed for PUUV-specific IgM and IgG by using a strip-immunoassay (*recomLine* Bunyavirus IgG/IgM, Mikrogen, Neuried, Germany).

Statistical Analysis

Laboratory findings were tabulated as medians and interquartile ranges (IQRs). Clinical symptoms were enumerated by percentage of affected patients.

Results

Clinical Findings and Course of Acute NE

During September 2012–April 2013, we contacted (by mail) 1,570 persons in Baden-Württemberg State,

Germany, for possible inclusion in this study; all of these persons had received a diagnosis of serologically confirmed NE during 2001–2012. Of the 1,570 persons, 456 (29%) were included in the study, representing 6.1% of ever-reported cases of hantavirus infection in Germany. Reasons for exclusion included failure to respond to the request (1,104 patients), being <18 years of age at the time of diagnosis (3 patients), and missed follow-up appointment (10 patients) (online Technical Appendix).

Table 1. Baseline characteristics for 456 persons during acute hantavirus infection, Germany, 2001–2012

Variable	Median value (range) or no. or % patients positive for variable
Sex, no.	
M	290
F	166
Age at diagnosis	48 y (40–59)
Patient status, no.	
Inpatient	335
Outpatient	121
Duration of hospital stay	7 d (5–9)
Duration of illness at hospital admission or first visit to general practitioner/nephrologist	5 d (3–7)
Duration of fever before hospital admission	4 d (2–6)
Duration of disease	4 wk (2–8)
Assumed exposure to virus, %	
Occupational	7
During leisure activities	93
Symptoms, %	
Pain	
Abdominal	33
Back	67
Limbs	71
Headache	68
Visual disorders	22
Diarrhea	20
Nausea/vomiting	47
Dyspnea	2
Loss of appetite	4
Fatigue	7
Shivering	2
Clinical signs	
Fever, %	90
Duration of fever	5 d (3–7)
Hemorrhage, %	7
Blood pressure	
Systolic,	130 mm Hg (120–146)
Diastolic	80 mm Hg (71–90)
Heart rate	76 beats/min (64–86)
Hypertension, %	15
Hypotension, %	10
Shock, %	1
Chest radiograph, %	47
Pathologic findings on radiograph, %	23
Abdominal ultrasound, %	65
Splenomegaly, %	14
Hepatomegaly, %	4
Acute kidney injury, %*	86

*According to RIFLE (risk of renal dysfunction) criteria (27).

Median age at diagnosis was 48 years (IQR 40–59). Of the 456 patients, 290 (64%) were male and 166 (36%) were female; the higher number of cases in men corresponds to the characteristics of the overall reported cases in Germany during 2001–2013 (Survstat, <https://www.survstat.rki.de/>). Of the 456 patients, 335 had received inpatient treatment, and 121 had received outpatient treatment by general practitioners or nephrologists. Time from onset of symptoms to admission to hospital or first contact with a general practitioner/nephrologist was 5 days (range 3–7). Prominent clinical findings were fever (90%), back pain (67%), limb pain (71%), and nausea and/or vomiting (47%). Baseline characteristics of the study population during the acute course of the disease are summarized in Table 1. At hospital admission/first visit to the general practitioner/nephrologist, 70% of the patients exhibited acute kidney injury, as determined by RIFLE (risk of renal dysfunction) criteria (27). Thrombocytopenia was present in 61% of patients at hospital admission/first visit with a general practitioner/nephrologist. Severe thrombocytopenia (platelet count $\leq 60 \times 10^9/L$) was found in 49 patients (11%), none of whom required platelet transfusion. Mild hemorrhagic symptoms, mostly epistaxis, occurred in 7% of the patients; no patients had severe bleeding complications. Lymphocytopenia was found in 55% of patients, monocytosis was observed in 59% at hospital admission/first visit to the general practitioner/nephrologist and C-reactive protein levels were substantially elevated in 96% of patients. Baseline characteristics are summarized in Table 1 and Table 2.

Follow-up

Baseline characteristics of the study population at the follow-up examination are summarized in Table 3. The median time between diagnosis with acute disease and follow-up was 17 months (IQR 7–35). Fifty per cent of patients had NE >2 years before the follow-up visit, and 20% had NE >5 years before the follow-up visit. All but 2 patients had serum creatinine levels within the reference range at follow-up. One of these 2 patients had hypertension and coronary disease before onset of NE; the second patient received a diagnosis of glomerulonephritis 3 months after the onset of NE. Thrombocytopenia was still present in 4% of the patients at follow-up. Proteinuria was present in 34 patients (7%). None of the patients had symptoms of urinary tract infection at follow-up. Urinalysis showed a high proportion of patients with hematuria (26%). We compared baseline characteristics (e.g., laboratory values, severity of acute kidney injury, symptoms during acute course of the disease) and findings (e.g., kidney function, blood pressure, and protein levels in urine) from the acute course of the disease for patients with and those without hematuria at follow-up: no statistically significant differences were observed between the groups (data not shown).

Blood pressure was measured twice for all patients in the outpatient clinic. In this setting, 203 (45%) of the 456 patients had hypertension stage 1 or 2, according to the classification of blood pressure for adults (26). All of these patients were contacted again within 8 weeks after the appointment in the outpatient clinic to reevaluate blood pressure measurements in the ambulatory setting. On the basis of these data, hypertension was present in 23% of

Table 2. Laboratory results for serum samples obtained from 456 persons experiencing acute hantavirus-associated nephropathia epidemica, Germany, 2001–2012*

Laboratory test	Median value (range)	Reference value
Creatinine, serum, maximum	2.7 mg/dL (1.6–4.8)	0.5–1.4 mg/dL
Platelet count		$>150 \times 10^9/L$
At admission or first visit with a general practitioner/nephrologist	122 (81–197)	
Minimum	113 (75–189)	
At discharge§	281 (226–353)	
Hematocrit		0.37–0.47
At admission or first visit with a general practitioner/nephrologist	0.42 (0.39–0.47)	
At discharge	0.40 (0.36–0.43)	
C-reactive protein		0.1–0.4 mg/dL
At admission or first visit with a general practitioner/nephrologist	4 mg/dL (2.4–7.1)	
Maximum	4.5 mg/dL (2.8–8.2)	
At discharge§	0.9 mg/dL (0.4–1.7)	
Liver enzyme†		
Aspartate aminotransferase		<50 U/L
At admission or first visit with a general practitioner/nephrologist	36 U/L (27–55)	
At discharge§	51 U/L (30–71)	
Alanine aminotransferase		<50 U/L
At admission or first visit with a general practitioner/nephrologist	35 U/L (24–57)	
At discharge§	64 U/L (35–101)	
Lactate dehydrogenase‡	273 U/L (240–323)	<250 U/L

*All patients were adults who had received a diagnosis of serologically and clinically confirmed hantavirus infection.

†35% of patients had elevated levels of liver enzymes at admission or first visit with a general practitioner/nephrologist.

‡66% of patients had elevated levels of lactate dehydrogenase at admission or first visit with a general practitioner/nephrologist.

§335 patients who received in-patient treatment.

Table 3. Baseline characteristics for 456 participants in a follow-up study to determine the clinical course and long-term outcome of hantavirus-associated nephropathia epidemica, Germany, 2001–2012*

Variable	Median value (range) or % patients positive for variable	Reference value
Laboratory test		
Creatinine, serum	0.9 mg/dL (0.8–1.0)	0.5–1.4 mg/dL
Blood cell counts		
Leukocytes	$6.7 \times 10^9/L$ (5.7–7.9)	$4.0\text{--}11.3 \times 10^9/L$
Platelets	$231 \times 10^9/L$ (201–268)	$>150 \times 10^9/L$
Hemoglobin	14.9 g/dL (14.1–15.6)	13–18 g/dL
Hematocrit	0.43 (0.42–0.46)	0.37–0.47
Uric acid	5.2 mg/dL (4.3–6.0)	4.8–5.6 mg/dL
Liver enzymes		
Aspartate aminotransferase	21 U/L (17–25)	<50 U/L
Alanine aminotransferase	21 U/L (15–30)	<50 U/L
Lactate dehydrogenase	158 U/L (142–180)	<250 U/L
C-reactive protein	0.1 mg/dL (0.1–0.2)	0.1–0.4 mg/dL
Urinalysis		
Proteinuria	7	–
Hematuria	26	–
Leukocyturia	15	–
Presence of bacteria	0	–
Hantavirus-specific antibodies		
IgM present	9	–
IgG present	100	–
Clinical test		
Blood pressure†		
Systolic	135 mm Hg (125–148)	–
Diastolic	83 mm Hg (76–90)	–
Hypertension at first follow-up, 203/456 participants		
Stage 1 hypertension, 98/203 hypertensive patients	45	–
Stage 2 hypertension, 105/203 hypertensive patients	48	–
Hypertension at second follow-up up‡		
Stage 1 hypertension	23	–
Stage 2 hypertension	66	–
Heart rate	70 beats/min (59–79)	–
Use antihypertensive drugs	43	–
Family history of hypertension	60	–
Smoke cigarettes/cigars	33	–
Concomitant condition		
Coronary heart disease	4	–
Peripheral arterial disease	1	–
Heart failure	2	–
Diabetes mellitus	3	–

*Study participants were adults who had received a diagnosis of serologically and clinically confirmed hantavirus infection during 2001–2012 and who later (7–35 mo) participated in a follow-up study. –, not applicable.

†Hypertension stages 1 and 2 were defined according to the classification of blood pressure for adults (26).

‡Includes only those participants who had hypertension at the first follow-up visit. Retesting was done within 8 wk after first follow-up visit.

all patients at follow-up. One third of these hypertensive patients had documented preexisting hypertension. In patients with preexisting hypertension, there was no change in blood pressure after NE, and antihypertensive therapy was not increased to achieve pre-NE blood pressure values.

Serum samples from all patients were analyzed for hantavirus-specific IgM and IgG at follow-up. All patients had detectable IgG, and 8.5% had persistent IgM.

Discussion

The data from our study show that NE is responsible for severe acute kidney injury in a high proportion of patients with PUUV infection. Proteinuria and hematuria were frequently present during the acute course of NE. In contrast to findings in previously published small studies (18,19),

our findings show that proteinuria and hypertension are not long-term sequelae of the disease. Of note, hematuria was present during the acute course of disease and at long-term follow-up (median 17 mo [IQR 7–35 mo]). Furthermore, our data show that neutralizing antibodies are still present up to years after infection, and no recurrent disease was reported. During the acute course of PUUV infection, the disease causes acute kidney injury in 86% of patients. In our study, only 3% of the patients required transient renal replacement therapy; this percentage is lower than that reported in previous smaller studies (8,10,13–16). Compared with previous studies, our study used well-defined, consistent classification criteria to describe the clinical course of patients with NE (10,28,29). Apart from acute kidney injury, NE is clinically characterized by thrombocytopenia

and, frequently, proteinuria (8,9,30,31). Thrombocytopenia was present in 219 61% of the patients in our study, and severe thrombocytopenia (platelet count $\leq 60 \times 10^9/L$) was found in 49 (11%) patients. Of interest, none of the patients required platelet transfusion, and only mild hemorrhagic symptoms, mostly epistaxis, occurred in a small percentage (7%) of all patients, and the hemorrhagic symptoms occurred independently of thrombocyte counts. Previous smaller studies reported higher rates of bleeding complications in patients with NE (32,33). At hospital admission, the thrombocyte nadir had been reached in 90% of the patients; this finding supports the hypothesis that thrombocytopenia occurs in early stages of the disease. Furthermore, visual disorders were present in 22% of our study population; the disorders, caused by a swelling of the lens, are often one of the first symptoms of NE (34). Therefore, NE should be considered in patients with acute onset of blurred vision and fever, especially in areas where the disease is endemic.

Reports on the long-term outcome of NE in large cohorts of patients are lacking. Hypertension has been discussed as a long-term consequence of NE (17), and 2 previous follow-up studies of 36 patients in Finland (5 and 10 years after infection) reported that patients exhibited increased urinary protein excretion, glomerular hyperfiltration, and elevated blood pressure at the 5- but not the 10-year follow-up (18,19). Some reports have suggested an association between previous hantavirus infection and subsequent hypertension (20–23). In our study population, 23% of patients had hypertension at follow-up. It is noteworthy, however, that these patients were older (45–63 years of age) than the patients without hypertension. Furthermore, the incidence of high blood pressure in patients in our study was not different than that in an age-matched cohort of patients without a history of NE (35). In contrast to findings in previous studies (20–23), we did not find that hypertension was a frequent sequela of NE in our study population. Persistent proteinuria following NE is a currently unresolved but frequently discussed issue. In our study, only 30 patients exhibited proteinuria at follow-up, and 19 (57%) of these patients had received a diagnosis of hypertension before the development of NE, suggesting that proteinuria does not seem to be long-term consequence of NE. At follow-up, a large proportion of patients had excess traces of blood in the urine (i.e., microscopic hematuria). Whether persistent hematuria after NE reflects lasting damage to glomerular cells, as a long-term consequence, remains uncertain because of the lack of renal biopsy specimens at long-term follow-up.

Representative data on humoral immune responses to PUUV in a large cohort of patients are lacking (36). Hantavirus-specific IgM and IgG were detected at initial diagnosis and during follow-up. All patients had hantavirus-specific IgG antibodies at follow-up, suggesting

that neutralizing antibodies are still present up to years after infection.

Our study design had several limitations. First, only one third of NE cases occur with typical clinical signs and symptoms, which results in high underreporting, especially of younger patients with mild disease. Second, patients were contacted by mail and asked to attend the outpatient clinic for follow-up investigations, which may have led to a selection bias because more patients with a severe course of disease might have been included in the study. Third, we did a retrospective study of medical case reports for the patients during acute NE, associated with all known limitations. Previously published Finnish long-term outcome studies of patients with NE (18,19) investigated small cohorts of patients, but they provided accurate glomerular filtration rate measurements and 24-hour ambulatory blood pressure measurements and compared their results with those of a control group. Because of the large number of patients in our study and the wide geographic area in which they resided, we could not obtain 24-hour blood pressure monitoring; thus, we focused on blood pressure measurements obtained in the outpatient clinic at follow-up and on interviews with patients who had elevated blood pressure at that time. Therefore, establishment of a matched control group was not possible. The last possible limitation is that serum samples from PUUV-infected patients cross-react strongly with Sin Nombre virus and weakly with Hantaan virus, Seoul virus, and Dobrava virus (36,37). Although Dobrava-Belgrade virus and Tula virus circulate in rodent hosts in Germany and might cause an infection in humans (38–40), almost all hantavirus infections (especially in southern Germany) are caused by PUUV (5,7). This fact minimizes the risk for inclusion in our study of patients with HFRS caused by a hantavirus other than PUUV.

In summary, PUUV-associated NE is responsible for acute kidney injury in a high percentage of patients. Hypertension and proteinuria do not seem to be long-term consequences of NE, but hematuria may be, and patients should therefore be monitored after PUUV infection. The presence of hantavirus-specific IgG in patients after PUUV infection suggests that neutralizing antibodies can be present as long as years after infection.

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The image shows a screenshot of a Twitter profile for CDC_EIDjournal. The profile bio reads: "EMERGING INFECTIOUS DISEASES is a peer reviewed, open access journal published monthly by CDC. cdc.gov/eid". The profile statistics show 30 tweets, 40 following, and 85 followers. The tweets section shows two tweets from CDC_EIDjournal@CDC_EIDjournal, one about a podcast on horses and another about a report on Bartonella species. A text overlay at the bottom of the screenshot says: "Sign up for Twitter and find the latest information about **emerging infectious diseases** from the EID journal." Below this text is a Twitter bird icon and the handle @CDC_EIDjournal.

Detection of Zika Virus in Urine

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Elodie Calvez, Cyrille Goarant,
and Myrielle Dupont-Rouzeyrol

We describe the kinetics of Zika virus (ZIKV) detection in serum and urine samples of 6 patients. Urine samples were positive for ZIKV >10 days after onset of disease, which was a notably longer period than for serum samples. This finding supports the conclusion that urine samples are useful for diagnosis of ZIKV infections.

Zika virus (ZIKV) is an emerging mosquito-borne pathogen (family *Flaviviridae*, genus *Flavivirus*) that was isolated in 1947 from a rhesus monkey in the Zika forest in Uganda (1). ZIKV is believed to be transmitted to humans by infected *Aedes* spp. mosquitoes (2,3). Studies have demonstrated that ZIKV is endemic to Africa and Southeast Asia (4). Before 2007, few cases of human infection with ZIKV had been reported. In 2007, an epidemic of ZIKV infection in humans occurred in Yap, Federated States of Micronesia, in the Pacific region. A seroprevalence survey determined that $\leq 70\%$ of the population had been infected (5). During 2007–2013, the few cases of infection with ZIKV reported were in travelers returning from Africa (6) or Southeast Asia (7).

In humans, ZIKV infection is characterized by mild fever (37.8°C–38.5°C); arthralgia, notably of small joints of hands and feet; myalgia, headache; retroorbital pain; conjunctivitis; and cutaneous maculopapular rash. ZIKV infection is believed to be asymptomatic or mildly symptomatic in most cases (5). Thus, Zika can be misdiagnosed during the acute (viremic) phase because of nonspecific influenza-like signs and symptoms. Hemorrhagic signs have not been reported in ZIKV-infected patients (5–7). However neurologic complications, including Guillain-Barré syndrome, have been observed (8).

Biological confirmation of ZIKV infections is based mostly on detection of virus RNA in serum by using reverse transcription PCR (RT-PCR). Although IgM against ZIKV can be detected by ELISA, few laboratories have this ability. Thus, in addition to the nonspecific clinical features of infection with ZIKV, laboratory diagnosis is challenging because of low viremia and cross-reactivity of ZIKV antibodies with other flaviviruses (including dengue), which require confirmation by neutralization assays (8) and make rapid serologic confirmation difficult. We investigated the

diagnostic utility of urine as a source for detection of ZIKV RNA by real-time RT-PCR.

The Study

In October 2013, a ZIKV outbreak was reported in French Polynesia (9). This was the second outbreak of ZIKV infection reported in the Pacific region. In New Caledonia, where ZIKV infection had never been documented, the first cases of ZIKV infection imported from French Polynesia were confirmed by the end of November, and the first autochthonous cases were reported by mid-January 2014. Early in February 2014, the New Caledonia Health Authority declared an outbreak situation. By the end of August 2014, >1,400 cases of ZIKV infection were biologically confirmed, including 34 cases imported from French Polynesia (10).

Written informed consent was obtained from all patients in this study. Clinical signs and symptoms of 6 ZIKV-infected patients are shown in the Table. In this study, a cutaneous maculopapular rash of the trunk and extremities was systematically observed and considered a relevant clinical criterion. Complete blood counts showed a discreet perturbation common in many viral infections (mild leukopenia and thrombocytopenia associated with activated lymphocytes).

To detect ZIKV in samples (RNA extracted from 200 μ L of serum or urine), we used both sets of primers/probe specific for ZIKV (11). A standard curve with serial dilutions of known concentrations of a ZIKV virus stock was used to estimate viral load in samples. All blood samples were also tested for dengue virus and chikungunya virus by real-time RT-PCR and showed negative results. ZIKV was detected in serum of 4 patients (Figure). Urine samples from 2 other patients were also positive for ZIKV, and showed a higher viral load than corresponding serum samples and were positive for ≤ 7 days (patient 4) and probably >20 days (patient 3) after viremia reached an undetectable level (Figure). Urine samples from 6 healthy patients were also assessed and showed negative results.

Partial sequences of the gene for ZIKV nonstructural protein were obtained (12) directly from amplification products from urine or serum samples. Sequences obtained (GenBank accession nos. KJ873160 and KJ873161) had 100% identity with the sequence of a ZIKV strain isolated from a patient who returned from French Polynesia in 2013. As observed previously (9,13), sequences also had 98% identity with sequences of ZIKV strains isolated in Cambodia in 2010 and in Yap in 2007.

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Table. Characteristics of 6 patients infected with Zika virus, New Caledonia, 2014*

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Clinical						
Headache	–	–	–	–	+	–
Arthralgia	–	+++ (ankles)	++ (hands)	+++	++ (hands)	–
Maculopapular rash	+++	++	+++	++	+++	+++
Itching	+	+	+	++	+++	++
Edema in hands	+	–	–	++	–	++
Conjunctivitis	–	++	–	+	+	–
Adenopathy	++	–	–	–	–	–
Fever	+	+	+	++	++	–
Retroorbital pain	++	–	–	–	–	–
Myalgia	NA	–	+	+	+	+
Laboratory						
Leukopenia	–	–	+	–	NA	+
Thrombocytopenia	–	–	+	–	NA	–
Activated lymphocytes	+	+	+	+	NA	+

*–, negative; +, light; ++, moderate; +++, intense; NA, not available.

Conclusions

We report the suitability of urine samples for diagnosis of ZIKV infection by showing that ZIKV RNA is detectable in urine at a higher load and with a longer duration than in serum. ZIKV infection has been poorly described because it is a benign, self-limiting illness in most cases (5). Thus, ZIKV infection has probably been underdiagnosed and underreported in disease-endemic settings (4) or in returning travelers. However, if perifocal vector control is to be implemented and severe neurologic complications are to be avoided, biological confirmation of ZIKV infection is essential. Because of the absence of specific IgM-based diagnostic tests, molecular confirmation is the only method available for routine diagnosis.

For ZIKV infection, date of onset of illness is difficult to establish because of sporadic and frequently mild fever.

Although rash has been reported 3–5 days after the febrile phase (6,7), the 6 patients in our study had light asthenia and mild fever 2–3 days before the rash was observed; these symptoms were considered indicative of disease onset. Therefore, at the time the rash was observed, viremia was probably decreasing, which makes detection of virus in serum samples extremely challenging (Figure).

Other groups have reported that other flavivirus genomes, such as those of dengue virus (14), West Nile virus (15) and recently ZIKV (13), can be detected in urine samples for a longer time than in serum samples. Furthermore, use of urine samples for laboratory testing has some advantages, such as noninvasive sampling and ease of use. We detected ZIKV RNA in urine samples from all 6 patients. Urine samples showed strongly positive results; estimated maximum viral load was 0.7–220.10⁶ copies/mL.

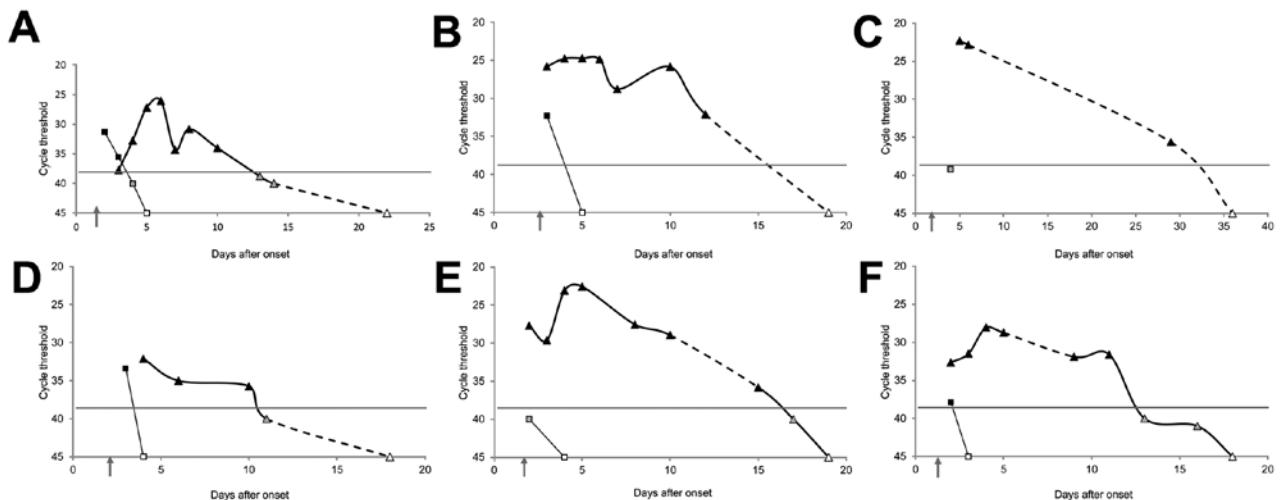


Figure. Detection of Zika virus in blood and urine specimens of 6 patients by using real-time reverse transcription PCR with primers/probe 1086/1162c/1107-Cy5 (11) New Caledonia, 2014. A) Patient 1; B) Patient 2; C) Patient 3; D) Patient 4; E) Patient 5; F) Patient 6. Triangles indicate urine samples and squares indicate serum samples. The cutoff cycle threshold (C_t) value is 38.5, as previously reported (11) and is indicated by horizontal lines. Black symbols indicate amplifications with $C_t < 38.5$, gray symbols indicate amplifications with $C_t \geq 38.5$, and white symbols indicate negative amplifications. Onset of disease (day 0) was defined retrospectively after questioning patients about initial symptoms. Dashed lines indicate a period >2 days without a sample being obtained. Arrows indicate onset of rash.

For all cases with sequential specimens, ZIKV RNA was detected ≤ 15 days (range 10 days to >20 days) after onset of symptoms, which was >7 days after it was not detected in serum samples.

In our study, ZIKV was detected in patient serum until a rash was observed (days 2–3 after disease onset). However, urine was preferred for virus detection. We observed a slight increase in ZIKV RNA from urine over the first few days after disease onset and rash (Figure). We therefore attempted to isolate ZIKV from urine samples, but failed to cultivate infectious particles. Further investigations are needed to evaluate whether live infectious ZIKV particles are excreted in urine, as has been observed for other arboviruses (15).

This study investigated the diagnostic utility of urine as a source for detection of ZIKV RNA by real-time RT-PCR. Results suggest that urine might be useful for confirmation of ZIKV infection because virus was detected at higher titers and for a longer period in urine samples than in serum samples. Although these results need confirmation in larger cohorts, they strongly suggest the suitability of urine as a specimen for ZIKV detection and screening in large-scale investigations or other epidemiologic contexts (e.g., returning travelers).

In industrialized regions, where local transmission of arboviruses, such as dengue virus or chikungunya virus has been reported, physicians should test patients who return from tropical regions for ZIKV when a case of dengue-like infection is suspected. Travelers might be a source of local transmission because *Ae. albopictus* mosquitoes are a competent vector for ZIKV (3).

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PODCAST

Breathing Valley Fever

Dr. Duc Vugia, chief of the Infectious Diseases Branch in the California Department of Public Health, discusses Valley Fever.

<http://www2c.cdc.gov/podcasts/player.asp?f=8631241>

Rapid Diagnostic Tests for Identifying Avian Influenza A(H7N9) Virus in Clinical Samples

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To determine sensitivity of rapid diagnostic tests for detecting influenza A(H7N9) virus, we compared rapid tests with PCR results and tested different types of clinical samples. Usefulness of seasonal influenza rapid tests for A(H7N9) virus infections is limited because of their low sensitivity for detecting virus in upper respiratory tract specimens.

On March 31, 2013, in China, novel avian influenza A(H7N9) virus infection was diagnosed in 3 persons (1). By October 2013, human infection with influenza A(H7N9) virus had reemerged; the number of cases in this second epidemic wave exceeded that of the first wave (before October 2013) (2). As of March 10, 2014, the virus had caused 379 human cases and 135 human deaths during both epidemic waves in China (2). Because the sensitivity of currently available rapid diagnostic tests (RDTs) for detecting virus in clinical specimens from patients with A(H7N9) virus infection remains largely unknown, we evaluated the sensitivity and specificity of 6 such tests available in China for detecting A(H7N9) virus in different types of clinical specimens from infected patients.

Novel avian influenza A(H7N9) virus has become the most prevalent avian influenza virus strain affecting humans in China. Shortly after the March 2013 outbreak, a real-time reverse transcription PCR (rRT-PCR) for detection of A(H7N9) virus was developed by the Chinese National Influenza Center (3). Although rRT-PCR is now considered the standard laboratory-based assay for detecting

influenza virus infections, because of its high sensitivity and specificity, it requires high-level laboratory expertise and might not be available in all locations. Thus, the usefulness of RDTs for detecting A(H7N9) virus infection requires assessment. The sensitivity of 6 RDTs has been evaluated in Australia by using a laboratory influenza A(H7N9) virus isolate shared by the Chinese National Influenza Center and the WHO Collaborating Centre for Reference and Research on Influenza in Melbourne, Australia (4). However, the suitability of RDTs for detecting A(H7N9) virus in clinical specimens from patients remains largely unknown. We therefore evaluated the sensitivity and specificity of 6 RDTs (Table 1) available in China for detecting A(H7N9) virus in different types of clinical specimens.

The Study

The RDTs varied according to detection mechanism, time to results, storage temperature, and shelf life. Of the 6 RDTs, 3 were designed to detect influenza A and B viruses, 2 influenza A virus only, and 1 specifically H7 virus (test names and manufacturer information provided in Table 1). We followed manufacturers' instructions and visually read the results. At the time of the study, 5 of the 6 tests had been approved for detection of seasonal influenza viruses in China, and approval was still pending for the Wondfo H7 test for A(H7N9). Since then, the Wondfo H7 test has been approved by the China Food and Drug Administration.

To evaluate detection limits of the RDTs, we propagated vaccine candidate A(H7N9) virus strain A/Anhui/1/2013 in MDCK cells and determined the mean 50% tissue culture infectious dose (TCID₅₀) per milliliter on the basis of at least 3 independent assays. Viruses were standardized to 1×10^7 TCID₅₀/mL and serially diluted 10-fold in phosphate-buffered saline. The detection limit for 3 RDTs was 10^3 TCID₅₀/mL and for 2 RDTs was 10^4 TCID₅₀/mL; 1 RDT could not detect A(H7N9) virus. The following 3 RDTs with the highest sensitivity were chosen for further evaluation of A(H7N9) in clinical specimens: Wantai FluA, Wondfo FluA, and Wondfo H7 (Table 1). The specimens tested were throat swab or sputum (including tracheal aspirates) collected from patients with suspected A(H7N9) virus infection since late March 2013, confirmed by rRT-PCR with primers and probes described previously (1,3), and stored at -80°C .

To compare the efficiency of RDTs for detecting A(H7N9) virus and seasonal influenza A viruses, we

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Table 1. Sensitivity of 6 RDTs for influenza A(H5N7) virus*

RDT	Test time,	Storage	Shelf life,	Detection method	Type of test	Detection limit, TCID ₅₀ /mL†
	min	temperature, °C	mo			
Wantai Flu A Dot-ELISA‡	20–30	2–8	5	Dot-ELISA	Well, cartridge	10 ³
Wondfo Flu A§	15	4–30	8	Colloidal gold	Well, cartridge	10 ³
Wondfo H7 Subtype¶	15	4–30	8	Colloidal gold	Well, cartridge	10 ³
BinaxNOW Flu A&B#	15	4–30	24	Colloidal gold	Test strip on card	10 ⁴
ClearView Flu A&B**	15	4–30	24	Colloidal gold	Test strip	10 ⁴
Kaibilibi Flu A&B††	15	4–30	18	Colloidal gold	Well, cartridge	ND

*ND, not detected at the highest tested viral concentration (1 × 10⁷ TCID₅₀/mL); RDT, rapid diagnostic test; TCID₅₀, 50% tissue culture infectious dose. Manufacturer information available at URL for each test.
†Detection limit of A/Anhui/1/2013 (H7N9) virus.
‡http://www.ystwt.cn/flu.html.
§http://www.wondfo.com.cn/English/products/List.aspx?MenuID = 050402&ID = 124&temp = 4.
¶Antigen detection kit for human infection with the H7 subtype avian influenza virus, Wondfo Biological Co., Ltd., Guangzhou, China: approved by China Food and Drug Administration after study.
#http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6132a4.htm.
**http://www.alere.com/ww/en/product-details/clearview-exact-influenza-a-and-b.html.
††http://www.jzmc.cn/content.asp?id=559.

also used RDTs and rRT-PCR to test seasonal influenza A(H3N2)-positive and A(H1N1)pdm09-positive throat swab samples collected during January–April 2012. rRT-PCR testing for seasonal influenza virus was conducted according to the World Health Organization protocol (5).

In total, 110 throat swab or sputum specimens from 53 A(H7N9)-infected patients and 115 A(H3N2) and 97 A(H1N1)pdm09 throat swab specimens were tested by using the 3 selected RDTs and rRT-PCR; each specimen was prepared and tested by all 4 assays at the same time. As cycle threshold (C_t) values increased, indicating lower levels of influenza virus in the clinical samples, the sensitivity of RDTs decreased significantly (Table 2). Viral load in throat swab specimens from A(H7N9)-infected patients was significantly lower than that from A(H1N1)pdm09- and A(H3N2)-infected patients (Figure 1).

We then further compared the sensitivity of RDTs for detecting virus in A(H7N9) specimens and seasonal influenza virus specimens with the same influenza A matrix gene C₁ intervals. We found that for specimens with C_t <25, RDT sensitivity for A(H7N9) specimens and seasonal influenza virus specimens was similar. However, for specimens with C_t >25, RDT sensitivity was significantly lower when A(H7N9) specimens were compared with seasonal influenza virus specimens with the same C_t interval. Overall, RDT sensitivity for detecting A(H7N9)

virus was significantly lower than that for detecting A(H1N1)pdm09 or A(H3N2) viruses (p<0.01). Wantai Flu A and Wondfo Flu A detection of A(H1N1)pdm09 and A(H3N2) viruses did not differ significantly (p>0.05). According to the Wondfo H7 subtype colloidal gold kit, 56 (51%) of the 110 A(H7N9) samples were positive and all 212 A(H1N1)pdm09 (n = 97) and A(H3N2) (n = 115) samples were negative (Table 2), demonstrating that this RDT can distinguish between clinical specimens positive for A(H7N9) and seasonal influenza viruses and that its rate of positivity for detecting A(H7N9) viruses is higher than that of the other 2 RDTs tested (Table 2). Ten throat swab samples that were influenza virus negative by rRT-PCR were also negative by the 3 RDTs.

Considering that most A(H7N9) virus-infected patients had pneumonia and that the virus replicates more efficiently in the lower respiratory tract than in the upper respiratory tract (6,7), A(H7N9) viral loads are probably higher in specimens from the lower respiratory tract. Viral loads were significantly higher in sputum/tracheal aspirates than in throat swab samples collected at the same time (Figure 2).

Conclusions

Although most RDTs examined in this study detected not only seasonal influenza virus but also A(H7N9) virus, the sensitivity of RDTs was lower for A(H7N9) virus than for

Table 2. RDT positivity rates for detection of different influenza A virus subtypes in real-time reverse transcription PCR–positive specimens*

C _t	Wantai Flu A Dot-ELISA‡			Wondfo Flu A test, colloidal gold method†			Wondfo H7 Subtype test, colloidal gold method		
	H7N9	H1N1 pdm09	H3N2	H7N9	H1N1 pdm09	H3N2	H7N9	H1N1 pdm09	H3N2
<25	7/7 (100)	12/14 (86)	22/24 (92)	7/7 (100)	12/14 (86)	22/24 (92)	7/7 (100)	0/14 (0)	0/24 (0)
25–30	18/38 (47)	35/54 (65)	35/55 (64)	11/38 (29)	23/54 (43)	23/55 (42)	28/38 (74)	0/54 (0)	0/55 (0)
>30	13/65 (20)	8/29 (28)	15/36 (42)	6/65 (9)	7/29 (24)	15/36 (42)	21/65 (32)	0/29 (0)	0/36 (0)
Total	38/110 (35)	55/97 (57)	72/115 (63)	24/110 (22)	42/97 (43)	60/115 (52)	56/110 (51)	0/97 (0)	0/115 (0)

*Values are no. specimens positive by RDT/no. specimens positive by real-time reverse transcription PCR (%). C_t, cycle threshold; RDT, rapid diagnostic test.

†Sensitivity for influenza A (H7N9) virus was significantly lower than that for either H1N1 pdm09 or influenza A (H3N2) viruses (p<0.01, χ² test), but no statistically significant difference in sensitivity was found between A(H1N1)pdm09 and influenza A (H3N2) viruses.

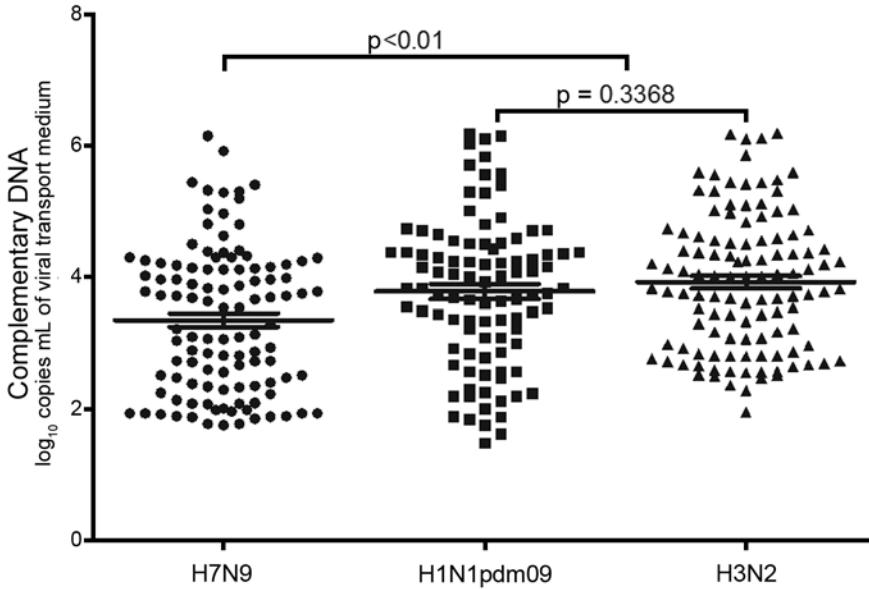


Figure 1. Viral loads of throat swab specimens collected from persons with avian influenza A(H7N9) and seasonal A(H3N2) and A(H1N1)pdm09 virus infection. Statistical analyses were performed by using a 1-way analysis of variance for the 3 groups and an unpaired *t*-test for comparison between the 2 seasonal influenza virus groups. Horizontal lines indicate medians and 95% CIs (above and below means).

seasonal influenza virus. Even for specimens with the same C_t intervals, RDT sensitivity to A(H7N9) virus was significantly lower than that for either A(H1N1)pdm09 or A(H3N2) virus. The most likely explanation is that cross-reactivity with the nucleocapsid protein-specific antibodies used in RDTs to detect seasonal influenza A virus was significantly lower for A(H7N9) virus. A previous study also indicated that detection sensitivity for swine-origin A(H1N1) viruses varies widely among seasonal influenza A virus RDTs; some tests are unsuitable for detecting several subtypes of avian influenza viruses because of low sensitivity (8).

The Wondfo H7 RDT evaluated in this study was based on a pair of anti-H7 monoclonal antibodies. We found that

for each of the 3 C_t intervals, the sensitivity for detecting A(H7N9) was relatively higher for the subtype H7 RDT than for the other RDTs.

Our study indicates that throat swab samples, which have been widely used for influenza diagnosis in China, are not suitable for RDT detection of A(H7N9) virus because of the low levels of virus they contain (Figure 1). Viral loads are significantly higher in sputum samples/tracheal aspirates from the lower respiratory tract than from throat swab samples (Figure 2). If any previously designed influenza A virus-specific RDTs are to be used for detection of A(H7N9) viruses, the kits should be modified for use with sputum and tracheal aspirates by improving extraction. In

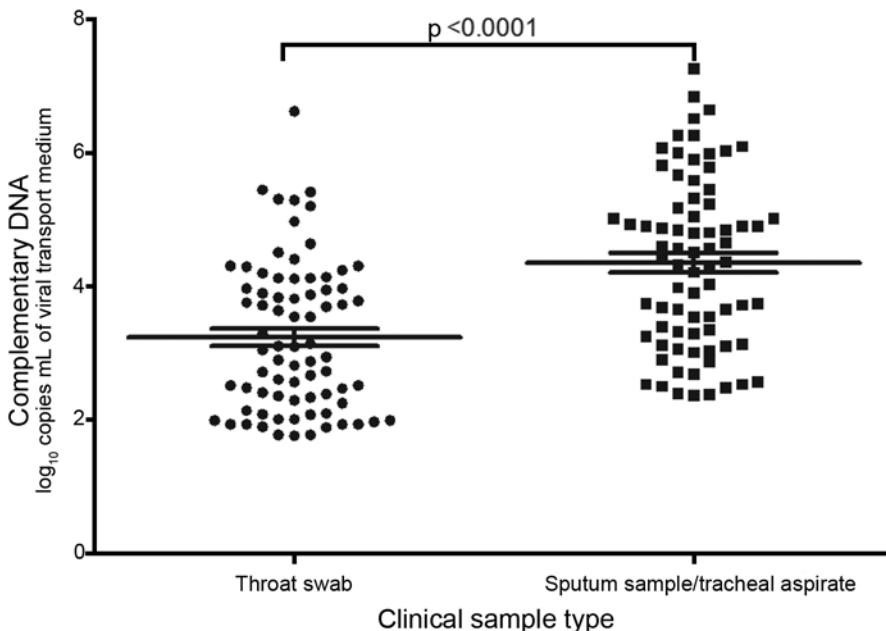


Figure 2. Comparison of viral loads of throat swabs and sputum specimens collected at the same time from persons with influenza A(H7N9) virus infection. Statistical analyses were performed by using a paired *t*-test. Horizontal lines indicate the medians and 95% confidence intervals (above and below means).

summary, usefulness of currently available seasonal influenza RDTs for diagnosing A(H7N9) virus infections is limited because of their low sensitivity for detecting virus in upper respiratory tract specimens.

Acknowledgments

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Acute *Blastocystis*-Associated Appendicular Peritonitis in a Child, Casablanca, Morocco

Emilie Fréalle,¹ Dima El Safadi,¹ Amandine Cian, Estelle Aubry, Gabriela Certad, Marwan Osman, Agnès Wacrenier, Emmanuel Dutoit, Colette Creusy, François Dubos, and Eric Viscogliosi

Despite increasing reports that *Blastocystis* infection is associated with digestive symptoms, its pathogenicity remains controversial. We report appendicular peritonitis in a 9-year-old girl returning to France from Morocco. Only *Blastocystis* parasites were detected in stools, appendix, peritoneal liquid, and recto-uterine pouch. Simultaneous gastroenteritis in 26 members of the child's family suggested an outbreak.

Blastocystis is a genus of anaerobic protozoan parasites that infect humans and a vast range of animal species. Prevalence in humans varies from 0.5%–24% in industrialized countries to 30%–76% in developing countries (1,2). Classic clinical features of infection include gastrointestinal symptoms such as nausea, anorexia, flatulence, and acute or chronic diarrhea. Fever is usually absent. An association with irritable bowel syndrome and extraintestinal manifestations such as urticaria has been suggested (2). Reports about invasive infection or disseminated diseases are rare (3). Here, we report the case of a pediatric patient infected with *Blastocystis* that was manifested by gastroenteritis associated with suppurative appendicitis and peritonitis.

The Study

In August 2013, a 9-year-old girl who was returning to France after a 1-month stay with her family in Casablanca, Morocco, was admitted to Lille University Hospital in Lille. Symptoms started in Casablanca 3 days before hospital admission and included fever, severe diarrhea (>10 liquid defecations/day), vomiting, and abdominal

pain in the hypogastric area and in the right and left lower quadrants associated with bilateral dorsal pain, anorexia, and weakness.

Blood count showed 13,850/mm³ leukocytes (75.4% neutrophils, 15.9% lymphocytes, 8.5% monocytes). C-reactive protein level was increased at 266 mg/L (Low risk: <1.0mg/L; average risk: 1.0–3.0 mg/L; high risk >3.0 mg/L). Traveler's gastroenteritis was diagnosed, and symptomatic treatment with acetaminophen, phloroglucinol glucoside, and acetorphan was prescribed. However, abdominal pain increased, and total food intolerance occurred in the following hours.

An abdominal ultrasound was performed, revealing appendicitis with suppuration in the recto-uterine pouch and a reflex ileus. Parasitologic examination of fecal matter revealed only abundant *Blastocystis* vacuolar forms, with >5 parasites per field. We further confirmed absence of *Cryptosporidium* spp. using glycerin assay and real-time PCR. Yeasts and a multimicrobial flora were present in the fecal material, but other infectious agents such as *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Yersinia enterocolitica*, adenovirus, and rotavirus were not detected. Similarly, multimicrobial flora, but no pathogenic bacteria, were detected in the peritoneal liquid and recto-uterine pouch (Table). Histopathologic observation revealed acute suppurative appendicitis with ulcerations extending deep into the muscularis, which was covered with a suppurative and fibrinous exudate. We observed infiltration by numerous neutrophils, eosinophils, plasma cells, and lymphocytes through all layers and into the serous membrane (Figure, panel A).

After hematoxylin-eosin staining and immunofluorescence labeling by using the anti-*Blastocystis* Paraflor B monoclonal antibody (Boulder Diagnostics, Marlborough, MA, USA), we detected parasitic forms in the lumen and in the lamina propria of the mucosa (Figure, panels B, C). We used real-time PCR for *Blastocystis* parasite detection as described (4), using DNA extracted from stools, appendix, peritoneal liquid, and the recto-uterine pouch, which all tested positive. We subsequently performed small subunit rDNA (SSU rDNA) amplification, then cloned the PCR product and sequenced 5 clones from all the DNA samples to subtype (ST) *Blastocystis* isolates and detect mixed infections (5). We identified ST3 in all the analyzed compartments. Mixed infection with ST2 and ST3 was detected only in the stools. The SSU rDNA gene sequences

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Table. Microbiological examination of samples of feces, appendix, recto-uterine pouch, and peritoneal fluid from child who had peritonitis, Casablanca, Morocco*

Variable	Date of sampling, 2013				
	Aug 30	Sept 1			Nov 13
Procedure/result	Feces	Appendix	Recto-uterine pouch	Peritoneal fluid	Feces
Microscopic examination	Numerous <i>Blastocystis</i> vacuolar forms; no <i>Cryptosporidium</i> or other parasites	Presence of rare <i>Blastocystis</i> †	ND	ND	Absence of parasites
Real-time PCR					
<i>Blastocystis</i> spp.	Positive	Positive	Positive	Positive	Negative
<i>Cryptosporidium</i> spp.	Negative	Negative	Negative	Negative	ND
Sequencing					
<i>Blastocystis</i> spp. genotype	ST2, ST3	ST3	ST3	ST3	ND
Bacterial culture	Negative for <i>Salmonella</i> , <i>Shigella</i> , <i>Campylobacter</i> , <i>Yersinia enterocolitica</i>	ND	Multimicrobial flora	Multimicrobial flora	ND
Viral antigen detection	Negative for adenovirus and rotavirus	ND	ND	ND	ND

*ND, not done; ST, subtype.

†Figure, panels B,C.

obtained in this study have been deposited in GenBank under accession nos. KJ605630–KJ605649.

The child completely recovered after an appendectomy, removal of a stercolith from the appendix lumen, and treatment with tinidazole, 20 mg/kg/d, and ceftriaxone, 50 mg/kg/d for 10 days, together with gentamicin, 5 mg/kg/d for 5 days. Although tinidazole is not the first line medication for treatment of *Blastocystis* infection, the child recovered completely and showed total clearance of parasites at day 73: using microscopy and real-time PCR on fecal samples, we found negative results for *Blastocystis*. Data obtained from the child's mother revealed simultaneous cases of gastroenteritis in 26 family members: 13 adults, 34–98 years of age, and 13 children, 18 months–15 years of age, who lived in the same building at the residential “Mohammadi” area of Casablanca. Adults had mild or moderate diarrhea but symptoms were more severe in children, who all had abundant diarrhea, vomiting, and weight loss. Repatriation in France of an 18-month-old baby was considered, but his condition improved. None of the family members required hospitalization. Unfortunately, no explorations were performed, therefore the diagnosis could not be microbiologically documented.

Conclusions

Reports of *Blastocystis* infection associated with diarrhea and clinical symptoms in immunocompetent and immunocompromised patients have increased during the past 2 decades (2). Tissue invasion of *Blastocystis* parasites in the appendix (6) or in the colon mucosa (3), associated with acute or chronic inflammation, has been reported. However, controversy still exists over whether this parasite is commensal or pathogenic; this case further supports its invasive and inflammatory potential. Previous reports regarding the presence of *Blastocystis* parasites in 4 of 100 appendix specimens from patients with acute appendicitis

(7), and of pseudoappendicular illness, which led to appendectomies in children with intestinal infection caused by this parasite (8), suggest that *Blastocystis* infection could be associated with appendicitis. Nevertheless, the actual role of *Blastocystis* in the pathogenesis of appendicitis remains inconclusive. In this report, the presence of a stercolith, which can be found in 50%–80% of appendicitis cases, suggested mechanical obstruction of the appendix's lumen, which is the main etiology of appendicitis.

Here, we report dissemination of *Blastocystis* into the lumen, the mucosa, and the recto-uterine pouch exudate, associated with appendicular acute inflammation, and no other infectious agent was detected. These observations, together with the well-documented acute or chronic inflammation occurring in humans or animals with *Blastocystis* infections (3,9), likely support the contribution of this infection to the inflammatory process. Infection with ST3 further reinforced this hypothesis. Indeed, the presence of pathogenic strains among ST3 has been confirmed through experimental infections in rats (9). Additionally, a substantial inflammatory reaction and an increased propagation of human colorectal cancer cells exposed to *Blastocystis* ST3 antigens has been demonstrated in vitro (10). For humans, the pathogenicity of different STs is unclear and remains a debatable issue. ST1 isolates were found to be more prevalent among symptomatic patients in Lebanon (5), but ST3 was found to be the only ST that showed pathogenic potential in Malaysian patients when compared with ST1 and ST2 (11). ST3 was also found to be significantly associated with diarrhea in Libya ($p = 0.008$) (12). For this case, the fact that only ST3 was detected in all analyzed samples, whereas a mixed infection with ST2 and ST3 was found in the child's stools, further supports the high invasive potential of ST3. ST3 is the most common ST in Europe, but, in African countries, its frequency varies from 17.8% in Libya (12) to 61.9% in Egypt (13). In Morocco, a 28.7% prevalence of blastocystosis has been

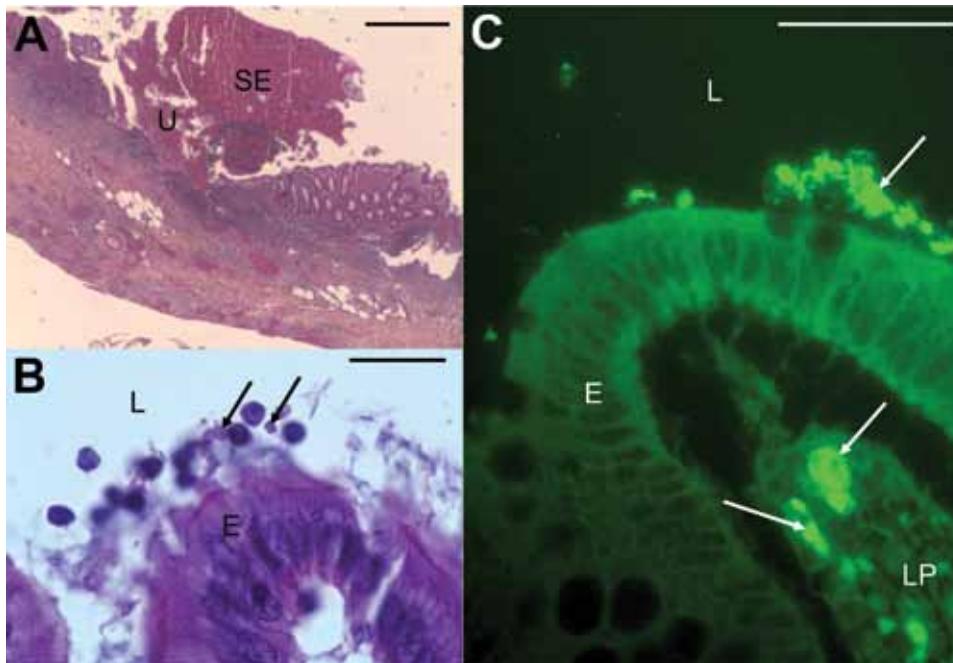


Figure. Micrographs showing histopathologic examination of appendix samples from a child who had peritonitis, Casablanca, Morocco, 2013. A) Ulceration (U) covered with suppurative and fibrinous exudates (SE) (hematoxylin-eosin stain). Scale bar indicates 200 μ m. B) *Blastocystis* parasites (arrows) in the lumen (L), and at the surface of the epithelium (E) (hematoxylin-eosin stain). Scale bar indicates 20 μ m. C) *Blastocystis* parasites (arrows) in the lumen (L), at the surface of the epithelium (E) and in the lamina propria (LP) of the mucosa (immunofluorescence labeling with anti-*Blastocystis* ParaFlorB antibody). Scale bar indicates 50 μ m.

reported, but data concerning the ST distribution of the parasite are not available (14). Furthermore, although *Blastocystis* infection could not be confirmed among the child's relatives, the simultaneous occurrence of gastroenteritis cases in the same family and the absence of other infectious agents in the child's stools suggest a potential outbreak of *Blastocystis* infection. *Blastocystis* parasites could have spread within the child's family, as previously reported in Italy, where 2 adopted children originating from India and the Côte d'Ivoire transmitted *Blastocystis* parasites to their adoptive parents and grandmother (15). Possible acquisition of this parasite from a common source such as contaminated water could also explain family transmission in this report. Altogether, these data highlight 1) the need for both systematic parasitologic examinations of stools in patients with invasive infections who are traveling from countries with high *Blastocystis* prevalence and 2) the need for routine provision of imidazoles for empiric treatment of peritonitis.

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Characterization of a Multidrug-Resistant, Novel *Bacteroides* Genomospecies

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Metronidazole- and carbapenem-resistant *Bacteroides fragilis* are rare in the United States. We isolated a multidrug-resistant anaerobe from the bloodstream and intraabdominal abscesses of a patient who had traveled to India. Whole-genome sequencing identified the organism as a novel *Bacteroides* genomospecies. Physicians should be aware of the possibility for concomitant carbapenem- and metronidazole-resistant *Bacteroides* infections.

We previously reported a 2013 case of intraabdominal abscesses and bacteremia caused by a multidrug-resistant anaerobe identified as *Bacteroides fragilis* (1). In brief, unremitting abdominal pain developed in a 71-year-old man who had been traveling in India for 1 month. The man was hospitalized locally and subsequently received a diagnosis of metastatic colon adenocarcinoma. He returned to Seattle, Washington, USA, for treatment consisting of 5 cycles of chemotherapy, followed by right hemicolectomy and right hepatectomy. On postoperative day 4, the patient showed marked leukocytosis, and abdominal abscesses were noted on computed tomographic scan images. Cultured percutaneous drainage fluid grew *Escherichia coli* that was resistant to ampicillin, trimethoprim/sulfamethoxazole, and fluoroquinolones. Therapy was then limited to ceftriaxone, and the patient's leukocyte count continued to rise and fever returned. Blood cultures grew anaerobic gram-negative rods identified as *B. fragilis* by MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometry and 16S rRNA sequencing. New rim-enhancing fluid collections in the abdomen and pelvis were noted on computed tomographic scan images, and percutaneous drainage fluid from these collections grew 3+ (moderate) quantities of *B. fragilis*. Isolates from blood culture and abscess fluid were resistant to multiple classes

of antimicrobial drugs, including metronidazole and imipenem (Table). The abscesses ultimately resolved after treatment for 60 days with linezolid and empiric ertapenem.

The Study

To better characterize the patient's clinical isolate, we subjected the organism to whole-genome sequencing by using the MiSeq platform (Illumina, San Diego, CA, USA). In brief, DNA was digested by using NEBNext dsDNA Fragmentase and then end-repaired and A-tailed by using *E. coli* DNA Polymerase I, T4 PNK, and Taq DNA Polymerase (all from New England Biolabs, Ipswich, MA, USA). Annealed Y adaptors (5'-(PO4-) GATCGGAAGAGCGGTTTCAG-CAGGAATGCCGAG-3' and 5'-ACACTCTTCCCTA-CACGACGCTCTTCCGATCT-3') were ligated by using T4 DNA Ligase in Rapid Ligation Buffer (Enzymatics, Beverly, MA, USA). The library was PCR-amplified with KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, USA) by using primer 1 (5'-AATGATACGGCGAC-CACCGAGATCTACTCTTTCCTACACGACGC-3') and primer 2 (5'-CAAGCAGAAGACGGCATAACGAGATCAAGGTCACGGTCTCGGCATTCTGCTGAACCG-3'). For sequencing, 250-bp paired-end reads were used with a custom index primer (5'-AGATCGGAAGAGCGGTTTCAGCAGGAATGCCGAGACCG-3'); sequencing was performed to an estimated coverage of 61× per base. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA, USA). De novo genome assembly was performed by using the ABySS v1.3.5 assembler (2); gene prediction and annotation, using the RAST server v4.0 (3); and comparative genomic analyses, using jSpecies v1.2.1 (4). The assembly was visualized by using BRIG 0.95 (5).

Initial comparison of the clinical isolate with the 3 completed *B. fragilis* reference genomes (638R, YCH46, NCTC 9343) showed a high degree of sequence divergence (Figure). We expanded our analysis to other sequenced *Bacteroides* species and observed similar results (Figure). An average nucleotide identity by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (ANiB) analysis (4) was performed (results are shown in online Technical Appendix Table 1, <http://wwwnc.cdc.gov/EID/article/21/1/14-0662-Techapp1.pdf>). Of note, pairwise ANiB values of <95% have been used as the cutoff for circumscribing species (4). The clinical isolate demonstrated pairwise ANiB values of 86.28%–86.54% against *B. fragilis* reference strains and

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Table. Antimicrobial susceptibility results for a novel *Bacteroides* genomospecies isolated from the bloodstream and intraabdominal abscesses of a patient with colon cancer, 2013

Antimicrobial drug	MIC, $\mu\text{g}/\text{mL}^*$
Ampicillin/subactam	>256/128
Cefotetan	64
Clindamycin	>256
Imipenem	>32†
Linezolid	2
Metronidazole	>256†
Minocycline	4
Moxifloxacin	>32
Piperacillin/tazobactam	>256
Synercid	>32
Tetracycline	16
Ticarcillin/clavulanic acid	>256/2
Tigecycline	1

*Antimicrobial susceptibility testing performed by using E-test (see online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-0662-Techapp1.pdf>).

†MICs confirmed as $\geq 64 \mu\text{g}/\text{mL}$ by macrobroth dilution.

even lower values compared with other *Bacteroides* species. In contrast, divergence among the 3 *B. fragilis* reference strains averaged 98.64% identity. These data are consistent with the conclusion that the isolate represents a genomospecies distinct from *B. fragilis*. This relationship was confirmed by using a BLAST search of the assembly against the nonredundant NCBI (National Center for Biotechnology Information) sequence database that contains published bacterial sequences; confirmation of the relationship indicated that the organism most closely resembles *B. fragilis* 638R and is not better classified as an alternative species already present in GenBank.

The clinical isolate contained an estimated 5.50 Mbp of DNA, $\approx 20\%$ more than sequenced *B. fragilis* reference strains, and 43.81% GC content. The shotgun sequence encodes 5,053 predicted genes, including 1,479 hypothetical proteins without inferred function. This number is significantly (1-tailed z -score = 1) larger than the average predicted gene content of *B. fragilis* reference genomes (average of 4,760 predicted genes), although we cannot rule out minor contributions from plasmid DNA. Of 1,696 *B. fragilis* core genes (those present in all *B. fragilis* reference strains), 1,508 (88.9%) were present in the clinical isolate.

We explored the basis of the isolate's antimicrobial resistance by performing a BLAST search of the assembly against a database of previously described known factors (6) (online Technical Appendix Table 2). Carbapenem resistance in *B. fragilis* has been shown to result from up-regulation of the *cfiA* metallo- β -lactamase (7), and we identified homologs of 2 β -lactamases, *cfxA* and *cfiA13*, in the clinical isolate (the latter of which had an upstream insertion sequence). Although the exact mechanism of metronidazole resistance is unknown, it has been attributed to chromosomally or plasmid-encoded nitroimidazole resistance (*nim*) genes encoding nitroimidazole reductase (8–10).

Although we did not detect homologs of canonical genes *nimA–J*, 2 putative nitroimidazole resistance genes were identifiable on the basis of functional annotation (10). We also detected *ermF* (macrolide resistance) and *tetQ* genes (tetracycline resistance). The isolate had a substitution in *gyrA* (Ser82 to Phe) known to confer resistance to fluoroquinolones (11). The draft genome (GenBank accession no. JANI000000000) and sequence reads (Sequence Read Archive accession no. SRP045260) are publicly accessible.

Conclusions

Members of the *Bacteroides* genus constitute a large fraction of the human gut microbiome and are important opportunistic pathogens that can cause a variety of serious infections. Metronidazole is thought to be almost universally effective against the species: only 1 of 1,957 *B. fragilis* clinical isolates collected across the United States during 2006–2009 was resistant to metronidazole (12). Similarly, previous studies have shown a prevalence of only $\approx 1\%$ of carbapenem-resistant *B. fragilis* (13), making this a favored second-line treatment (7). Nevertheless, there are limited but increasing reports of concomitant metronidazole and carbapenem resistance (14), and such resistance poses a threat to current treatment algorithms.

Genomic sequencing revealed the isolate in this study to be a genomospecies related to, but distinct from, *B. fragilis*. Techniques widely accepted as highly accurate for bacterial identification (16S rRNA sequencing and MALDI-TOF mass spectrometry) incorrectly identified this organism as *B. fragilis* (1). To determine whether previous multidrug-resistant isolates identified as *B. fragilis* might instead represent this novel genomospecies, we compared ANiB values for other isolates with those of the clinical isolate in our study. ANiB values indicate that 2 of the multidrug-resistant *B. fragilis* isolates sequenced in an earlier report (10) actually appear to be members of the genomospecies reported here (online Technical Appendix Table 3). Furthermore, these data highlight the potential for whole-genome sequencing to improve diagnostic accuracy in microbial identification and isolate characterization. Although we detected several antimicrobial drug-resistance factors in this isolate, alternative mechanisms of resistance to carbapenems and metronidazole, including non-*cfiA*-mediated carbapenem resistance and overexpression of efflux genes, respectively, have been also described and may contribute to the high levels of resistance observed with this isolate.

The increasing prevalence of carbapenem-resistant *Enterobacteriaceae* worldwide poses a major public health concern, often requiring use of more toxic “last-line agents,” such as colistin and polymyxin B. Although rare thus far, multidrug-resistant *Bacteroides* infections present a similar challenge to the treating physician and have

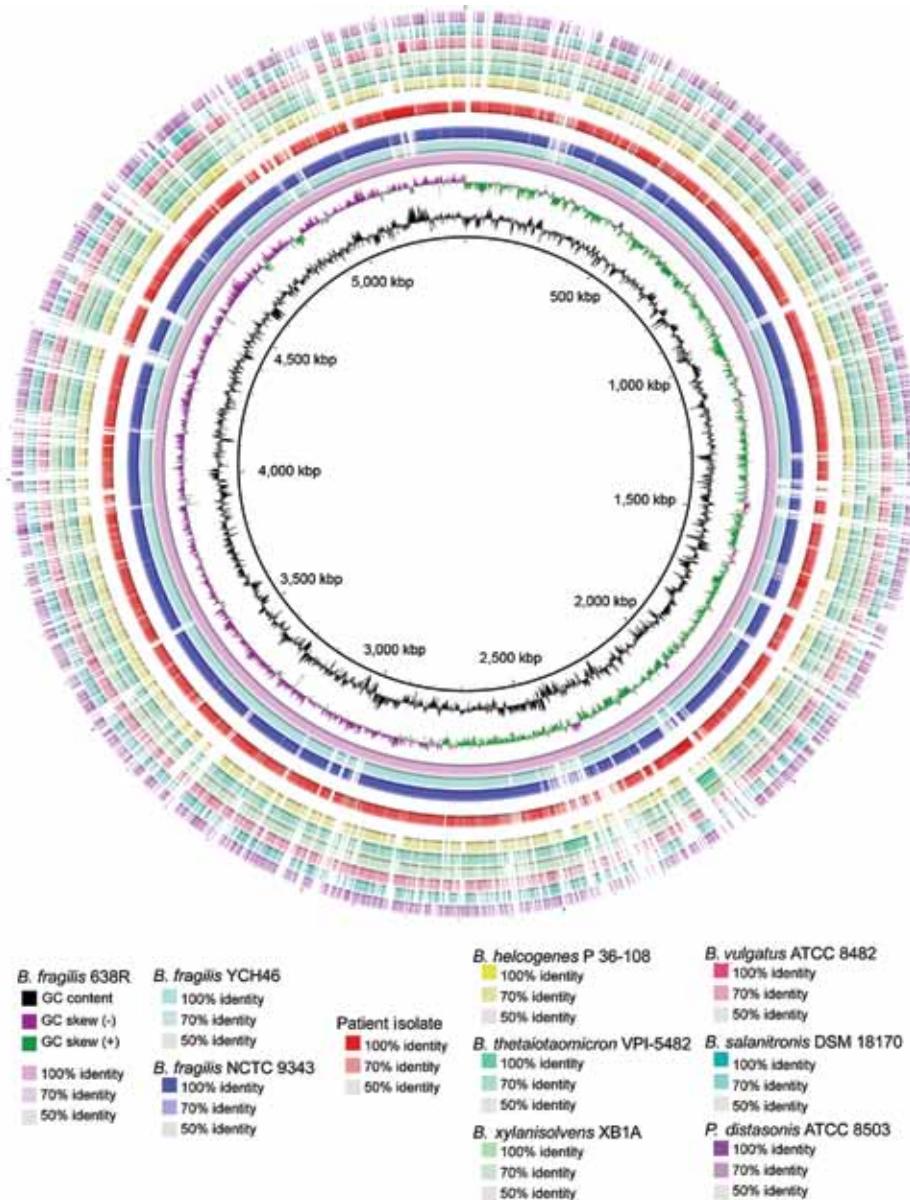


Figure. Characterization of circular plot of genome diversity between the clinical isolate of a multidrug-resistant, novel *Bacteroides* genomespecies and other *Bacteroides* spp. isolates. Reading from the center outwards, the map, GC content, and GC skew of the *B. fragilis* reference strain 638R are depicted. The white and colored regions of the following outer rings indicate regions absent and present, respectively, in genomes of the indicated organism compared with the genome of *B. fragilis* reference strain 638R. Intensity of coloration is proportional to the degree of sequence identity relative to the reference genome. The innermost 3 rings indicate the 3 *B. fragilis* reference genomes. The genome of the clinical isolate, separated from other rings by white space, follows. Non-*fragilis* *Bacteroides* species and a *Parabacteroides* species comprise the outermost rings. ATCC, American Type Culture Collection; DSM, Deutsche Sammlung von Mikroorganismen; NCTC, National Collection of Type Cultures.

the potential to become increasingly common in the future. Thus, providers should be aware of the possibility for concomitant carbapenem and metronidazole resistance in these organisms. Future studies will be required to determine what proportion of multidrug-resistant isolates can be ascribed to the novel genomespecies of *Bacteroides* reported here and whether this particular genomespecies offers any opportunities for targeted therapies.

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Enzootic Transmission of Yellow Fever Virus, Venezuela

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Phylogenetic analysis of yellow fever virus (YFV) strains isolated from Venezuela strongly supports YFV maintenance in situ in Venezuela, with evidence of regionally independent evolution within the country. However, there is considerable YFV movement from Brazil to Venezuela and between Trinidad and Venezuela.

Yellow fever virus (YFV) is the prototype species for the genus *Flavivirus*. Historically, YFV is one of the most important human arboviral pathogens. It continues to cause large sporadic epidemics in Africa but typically emerges as epizootics among nonhuman primates in South America with or without associated human cases (1–5). YFV emergence is cyclical; outbreaks occur ≈7–10 years apart. Several phylogenetic studies have shown that YFV is locally maintained during these interepizootic periods in Peru (6), Brazil (7), and Trinidad (4). These studies also have indicated that the virus undergoes regionally independent evolution within some countries (6).

YFV has caused sporadic outbreaks in Venezuela; the most recently documented epizootic/epidemic occurred in 2005 (8). Although endemic to Venezuela, YFV has very rarely been isolated and characterized, and partial sequences have been determined only for 4 strains. Venezuela is located between Trinidad and Brazil, which have contributed major evidence for the enzootic maintenance of YFV in South America. Thus, sequencing Venezuelan YFV strains over a wide geographic area and temporal distribution might be valuable to test the hypothesis of local maintenance in Venezuela and to determine whether the virus moves regularly between Trinidad and Venezuela or between Venezuela and Brazil. Understanding the maintenance and spread of YFV in South America also is critical for developing effective surveillance and prevention strategies.

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We sequenced a prM/E gene fragment of 10 YFV isolates from 4 locations within Venezuela, spanning 6 years (2004–2010; Table 1). Additionally, we sequenced complete genomes for 5 representative isolates for comparison with 12 previously determined genomic sequences (4,9).

The Study

The sporadic emergence of YFV in the Americas has been strongly associated with infection of red howler monkeys (*Alouatta seniculus*), which are particularly susceptible to disease. As exemplified in this study, nonhuman primate surveillance targeting this species remains an efficient strategy for monitoring enzootic YFV activity. Isolates made during a surveillance study aimed at investigating the ecology of infectious diseases in Venezuelan nonhuman primates were detected by cell culture and passaged once in Vero cells before sequencing, as previously described (4). Sequences were manually aligned in Se-AL (<http://tree.bio.ed.ac.uk/software/seal/>) and confirmed as nonrecombinant by using Recombination Detection Program (RDP4) (10). We obtained phylodynamic and phylogeographic estimates using Bayesian inference as implemented in BEAST v1.8.0 (11,12). We assessed the extent of geographic structuring using Bayesian tip-significance testing (13) based on the Markov chain Monte Carlo phylogenies estimated in BEAST. A Bayesian phylogeny was also inferred in Mr. Bayes (14) by using the general time reversible (GTR+I+ Γ_4) model for the complete open reading frame sequences.

Results of Bayesian tip-significance testing showed statistically significant geographic clustering among Venezuelan YFV strains. The association index, parsimony score, and maximum monophyletic clade statistics provided strong support ($p < 0.01$) that strains from Venezuela cluster by location, suggesting that YFV is maintained for long periods within Venezuela. Similar results have been shown for Peru (6), Brazil, and Trinidad (4). The high posterior probabilities, > 0.99 , observed at all nodes that delineate Venezuelan strains further support these conclusions (Figure 1).

We also found evidence of regionally independent evolution within Venezuela, as indicated by the existence of 2 phylogenetically distinct Venezuelan clades with posterior probabilities > 0.99 (Figures 1, 2). The clade containing a 2004 strain (2A) from Guárico and a 2010 strain (10A) from Monagas represented all but one of the sequences from eastern Venezuela (i.e., east of Caracas; Table 1). The 7 other strains were collected on the western

Table 1. Yellow fever virus strains sequenced in the study and their metadata, Venezuela*

Isolate ID	Source	Location	Year of collection	Passage history	GenBank accession no.
1A	Red howler monkey	Monagas	2004	Vero 2	KM388819
2A†	Red howler monkey	Guárico	2004	Vero 2	KM388817
3A	Red howler monkey	Portuguesa	2005	Vero 2	KM388820
4A	Red howler monkey	Portuguesa	2005	Vero 2	KM388821
5A	Human	Portuguesa	2005	Vero 2	KM388822
6A†	Human	Portuguesa	2005	Vero 2	KM388814
7A	Human	Portuguesa	2005	Vero 2	KM388823
8A†	Red howler monkey	Barinas	2006	Vero 2	KM388818
9A†	Red howler monkey	Apure	2007	Vero 2	KM388815
10A†	Red howler monkey	Monagas	2010	Vero 2	KM388816

*ID, identification. Red howler monkey, *Alouatta seniculus* species.

†Complete genome sequences determined.

side of Venezuela (including Portuguesa, Apure, and Barinas States) (Table 1). Two YFV strains were collected in 2004 from eastern and western Venezuela. Despite their nearly synchronous collections in 2004, these sequences fell into distinct clades in the maximum clade credibility (MCC) phylogeny, indicating population subdivision. Although we cannot rule out sampling bias, these data suggest in situ evolution of YFV in Venezuela and regionally independent evolution in distinct geographic foci within the country. The mechanism promoting this population subdivision among YFV strains is unclear and requires further investigation. Further studies on the ecology of the areas where these viruses were isolated might help explain the observed population subdivision.

Although YFV had been maintained in situ for several years within Venezuela, our phylogeographic results indicated YFV movement between Brazil, Trinidad, and

Venezuela. Brazil is the major source of YFV introductions into Venezuela, accounting for introductions of Venezuelan strains sampled in 1959, 1961, 1998, and more recently (i.e., independent introductions in 2004 in eastern and western Venezuela; Figure 1). The basal location of a 2004 sequence from the Brazilian Amazon within the western Venezuelan 2004–2007 clade (Figure 1) suggests that the 2005 Venezuelan epizootic/epidemic was initiated by an imported progenitor from the Brazilian Amazon Basin that later evolved independently within Venezuela. Our estimate of the most recent common ancestor for the node containing the 2004–2007 Venezuelan sequences was 2001 (95% highest posterior density 1997–2003), suggesting that the ancestral lineage existed for ≈ 3 years in Venezuela before its detection in nonhuman primates in 2004.

The 2004 Venezuelan (1A) intermediary descendent between the 1995 and 2009 Trinidad sequences is noteworthy.

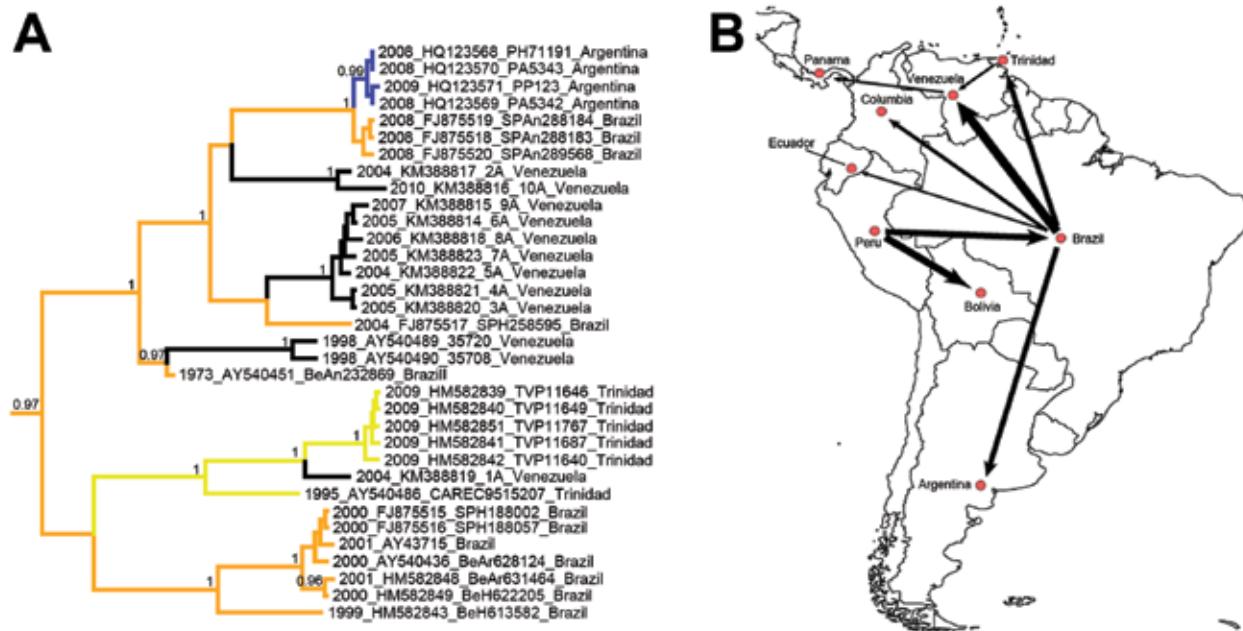


Figure 1. A) Magnified inset of the MCC phylogeny showing the tree topology for a subset of South American genotype I strains. B) Bayes factor (BF) test for significant nonzero rates indicating the statistical support for epidemiologically linked countries. Rates supported by a BF >5 are shown. The thickness of the arrows represents the relative strength by which the rates are supported. The Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/14-0814-Techapp1.pdf>) presents the details of the 124 sequences used in this study.

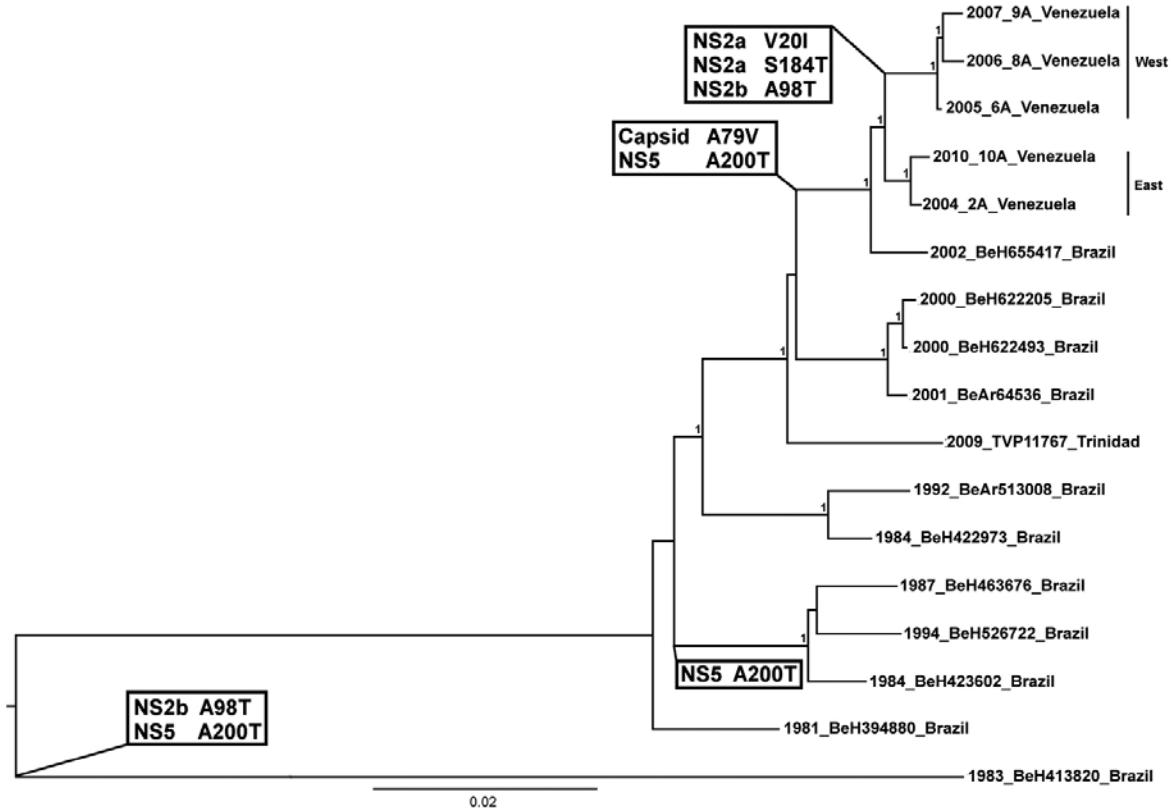


Figure 2. Midpoint rooted Bayesian Markov chain Monte Carlo phylogeny based on yellow fever virus (YFV) complete open reading frame sequences. Numbers at nodes indicate posterior probabilities ≥ 0.9 . Eastern and western Venezuelan sequences are indicated. Substitutions resulting from nonsynonymous, synapomorphic mutations that define sequences in a clade/lineage are highlighted at relevant nodes. Two substitutions (NS2b A98T and NS5 A200T) occurred in earlier isolates from Brazil. The capsid A79V and NS5 A200T substitutions include the Brazilian isolate BeH655417, which lies directly basal to the Venezuela isolates. This indicates that these substitutions were probably present in the YFV progenitor when it was introduced into Venezuela. Furthermore, substitutions NS2a V10I, NS2a S184T, and NS2b A98T all appear to have arisen after YFV was introduced to Venezuela, further supporting enzootic YFV maintenance there. Taxon/tip labels include year of isolation, strain names and country where the virus was isolated. Scale bar indicates percentage of nucleotide sequence divergence.

This cluster of Trinidad isolates was previously used as evidence of enzootic YFV maintenance within Trinidad during interepizootic periods (4). Incorporating our new sequences now shows movement between Trinidad and eastern Venezuela. In our phylogeny, strain 1A is the sister lineage of the 2008–09 Trinidad epizootic strains, with the 1995 Trinidadian

isolate lying basal to these sequences, with high posterior probabilities (Figure 1). The position of the 2004 Venezuelan 1A sequence possibly reflects importation from Trinidad, which implies that enzootic YFV circulation in Trinidad is not isolated epidemiologically but gave rise to exportation of YFV to Venezuela.

Table 2. Nucleotide and amino acid divergence among individual YFV genes of 9 representative YFV strains compared to the TVP11767* strain†

Genes	Strain, nucleotide (amino acid) divergence, %								
	BeH622205 (B, 2000)	BeAR51300 (B, 1992)	BeH423602 (B, 1984)	BeH413820 (B, 1983)	BeH394880 (B, 1981)	BeH655417 (B, 2002)	6A (V, 2005)	9A (V, 2007)	10A (V, 2010)
Capsid	98.3 (100)	98.0 (100)	97.2 (100)	91.2 (97.4)	96.3 (100)	97.2 (99.1)	97.2 (99.1)	97.2 (99.1)	96.6 (100)
PreM/M	97.7 (99.4)	96.7 (100)	91.2 (98.1)	97.3 (100)	97.3 (100)	96.2 (99.4)	97.7 (100)	97.7 (100)	97.7 (100)
E	98.2 (100)	96.3 (100)	89.6 (98.6)	96.9 (100)	97.5 (100)	96.6 (100)	97.2 (100)	97.0 (100)	97.6 (100)
NS1	97.9 (99.7)	97.3 (100)	90.5 (99.4)	97.5 (99.7)	98.1 (100)	96.8 (99.7)	98.7 (100)	98.4 (100)	98.6 (100)
NS2A&B	97.4 (100)	96.2 (99.4)	90.4 (99.4)	96.8 (100)	97.6 (100)	95.8 (99.1)	97.2 (100)	96.9 (99.4)	97.6 (100)
NS3	97.4 (100)	96.7 (100)	88.4 (100)	97.4 (100)	97.4 (100)	96.3 (100)	97.4 (100)	97.4 (100)	96.7 (100)
NS4A&B	98.2 (100)	96.8 (99.7)	90.3 (98.7)	97.1 (99.7)	97.8 (100)	97.1 (99.7)	98.1 (99.7)	98.0 (99.7)	98.2 (100)
NS5	98.3 (99.8)	96.6 (99.8)	91.1 (99.1)	96.8 (99.8)	98.0 (100)	96.6 (100)	98.4 (100)	98.1 (100)	98.2 (100)

*First South American complete genome sequence to be published. TVP11767 was isolated in 2009 from an *Alouatta seniculus* specimen in Trinidad. †YFV, yellow fever virus; B, Brazil; V, Venezuela; M, membrane; E, envelope; NS, nonstructural.

In the absence of more dense sampling, whether bidirectional YFV movement occurs between Venezuela and Trinidad is unclear. Given the proximity and boating traffic between these countries, substantial mixing between their YFV populations would not be surprising. Isolation and sequencing of additional YFV isolates from eastern Venezuela are needed to further evaluate movement between Trinidad and Venezuela.

Complete genomes were sequenced for 5 representative Venezuelan YFV strains from eastern and western Venezuela (Table 1). Comparison of nucleotide and amino acid similarities showed a high degree of conservation across YFV genes (Table 2). The 9 strains selected for comparisons represent the full spectrum of known YFV genetic diversity (Table 2; Figure 2). The most diverse genes shared >97% aa sequence identity, with >99.9% aa sequence identity for most proteins, even though these strains were collected >30 years apart (Table 2). The Bayesian Markov chain Monte Carlo phylogeny based on complete open reading frame sequences strongly supported the inferred maximum clade credibility tree, with all Venezuelan sequences grouping together with strong posterior support. The eastern and western Venezuelan strains grouped separately, with strong support in all of our inferred phylogenies. A total of 5 substitutions delineated the Venezuelan sequences (Figure 2).

Conclusions

Our phylogeographic analysis supports in situ evolution of YFV within Venezuela, as well as regionally independent evolution within the country. Brazil was identified as the major source of YFV introductions into Venezuela, and sequence analysis showed that considerable YFV movement may occur between Trinidad and Venezuela. Results of our Bayes factor test for non-zero rates also support the epidemiologic link between Venezuela, Brazil, and Trinidad (Figure 1, panel B). The sequences generated in our study fill a major gap in the geographic sampling of YFV.

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The GenBank accession numbers for the YFV sequences derived in this study are KM388814–KM388823.

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WU Polyomavirus in Respiratory Epithelial Cells from Lung Transplant Patient with Job Syndrome

Erica A. Siebrasse, Diana V. Pastrana, Nang L. Nguyen, Annie Wang, Mark J. Roth, Steven M. Holland, Alexandra F. Freeman, John McDyer, Christopher B. Buck, and David Wang

We detected WU polyomavirus (WUPyV) in a bronchoalveolar lavage sample from lungs transplanted into a recipient with Job syndrome by using immunoassays specific for the WUPyV viral protein 1. Co-staining for an epithelial cell marker identified most WUPyV viral protein 1–positive cells as respiratory epithelial cells.

WU polyomavirus (WUPyV) was discovered in a child with pneumonia in 2007 (1). Subsequent studies showed that WUPyV infection is common (2–4), and viral DNA can be detected in a variety of specimen types, including respiratory tract secretions (5). However, the virus has yet to be associated with any disease, and the specific cell type(s) infected by WUPyV has not been identified. Other human polyomaviruses are known pathogens, which typically cause disease in the context of immunosuppression (6–8).

Job syndrome is an immune disorder characterized by eczematoid dermatitis, recurrent skin and pulmonary infections, increased levels of IgE, and impaired T and B cell memory (9). This disorder is caused by dominant-negative mutations in the *STAT3* gene (9). We report WUPyV cell tropism in lungs transplanted into a recipient with Job syndrome.

The Study

These studies were approved by institutional review boards at the National Institutes of Health (NIH) and Washington University. A 28-year-old woman with Job syndrome was seen at the NIH Clinical Center 6 months after bilateral lung transplantation. She had a bronchoscopic evaluation to follow up on endobronchial aspergillosis. Pathologic examination

of a bronchoalveolar lavage (BAL) sample showed scattered cells, primarily columnar bronchial cells, with cytomorphologic changes reminiscent of BK polyomavirus (BKPyV)–infected decoy cells. The cells stained positive with PAb416, a monoclonal antibody against the SV40 large T antigen. The patient had BKPyV viremia (8.1×10^5 copies/mL) and viruria (6.9×10^9 copies/mL). JC polyomavirus was also detected in the urine but not in the blood. The BAL sample was weakly positive for BKPyV by PCR (<250 copies/mL) and negative for JC polyomavirus. Clinical or radiographic signs and symptoms of infection were not apparent.

Nonenveloped virions were purified from the BAL sample by using ultracentrifugation with Optiprep (#D1556; Sigma-Aldrich, St. Louis, MO, USA) (10). DNA was extracted from the virion preparation and subjected to random-primed rolling circle amplification (RCA) and restriction enzyme digestion, which yielded 2 strong bands. The bands were cloned and identified as WUPyV by using Sanger sequencing. The complete genomic sequence of the isolate, designated J1 (GenBank accession no. KJ643309), was confirmed by using miSeq analysis (Illumina, San Diego, CA, USA) of the RCA product. A second WUPyV variant with 2-nt polymorphisms and a single base insertion was also detected in the RCA product. No other known viruses (including BKPyV) were observed by deep sequencing.

We developed an immunohistochemical (IHC) assay to detect the WUPyV viral protein 1 (WU-VP1) by using an IgG2b designated NN-Ab06. Recombinant histidine-tagged WU-VP1 protein was generated by expressing WU-VP1 (GenBank accession no. ABQ09289) in *Escherichia coli* from a Gateway pDEST17 plasmid (Life Technologies, Carlsbad, CA, USA) and purifying the protein by using an affinity Ni-NTA column (Pierce Biotechnology, Rockford, IL, USA). After generation of hybridomas, we identified clones producing antibody against WU-VP1 by ELISA and immunoblot. Clones that cross-reacted with KI polyomavirus VP1 (KI-VP1) were identified by ELISA with glutathione S-transferase–tagged KI-VP1 (2) and eliminated.

To generate positive control cells for IHC assay optimization, we transfected 293T cells with plasmid pDEST26-WU-VP1 (Life Technologies). A subset of cells was fixed in 10% neutral-buffered formalin and embedded in paraffin. IHC testing was performed by deparaffinizing slides in xylene and rehydrating them in a series of ethanol solutions. After treating slides with 3% hydrogen peroxide, antigen was retrieved in citrate buffer, pH 6.0 (10 mmol/L citric

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acid, 0.05% Tween 20) in a pressure cooker (PC6–25; Nesco, Two Rivers, WI, USA) for 3 min on the high setting.

Slides were blocked in 1.5% normal horse serum (#S-200; Vector Laboratories, Burlingame, CA, USA) and incubated with NN-Ab06, then with biotinylated anti-mouse IgG (BA-2000; Vector Laboratories). After development by using the Vectastain Avidin–Biotin Complex Kit (#PK-6100; Vector Laboratories) and (3,3'-diaminobenzidine) (#SK-4100; Vector Laboratories), we counterstained tissues with hematoxylin.

Cells with prominent dark staining were seen (Figure 1, panel A). A serial section of the same cell block stained with an isotype-matched antibody (#557351, mouse IgG2b; BD Biosciences, San Jose, CA, USA) (Figure 1, panel B) and mock transfected cells stained with NN-Ab06 (Figure 1, panel C) showed negative results. Western blotting was performed as an independent means of evaluating specificity of NN-Ab06 (11). NN-Ab06 reacted with WU-VP1 protein lysate but not with KI-VP1 lysate, which is the most closely related virus to WUPyV. KI-VP1 has 65% amino acid identity with WU-VP1.

We applied the WU-VP1 IHC assay to formalin-fixed, paraffin-embedded sections of the BAL sample. Prominent dark staining of cells with enlarged nuclei and a ground glass appearance characteristic of viral cytopathic changes were observed (Figure 1, panel D). Staining was not seen in serial sections stained with the isotype antibody (Figure 1, panel E) or with no antibodies.

Many WUPyV-positive cells were cuboidal to columnar and showed other morphologic features consistent with respiratory epithelial cells. To determine their etiology, we developed a double immunofluorescence (dIF) assay with a polyclonal antibody against WU-VP1 (2), designated NN-Ab01, and a monoclonal antibody against cytokeratins (#M3515; Dako, Carpinteria, CA, USA). Deparaffinization and antigen retrieval were accomplished as noted above, and sections were blocked in Superblock T20 (#37516; Thermo Scientific, Waltham, MA, USA). To validate the assay, we performed immunofluorescence analysis with NN-Ab01 on positive control 293T cells expressing WU-VP1. The nucleus was counterstained with Hoechst (#H21491; Life Technologies). Several WU-VP1-positive

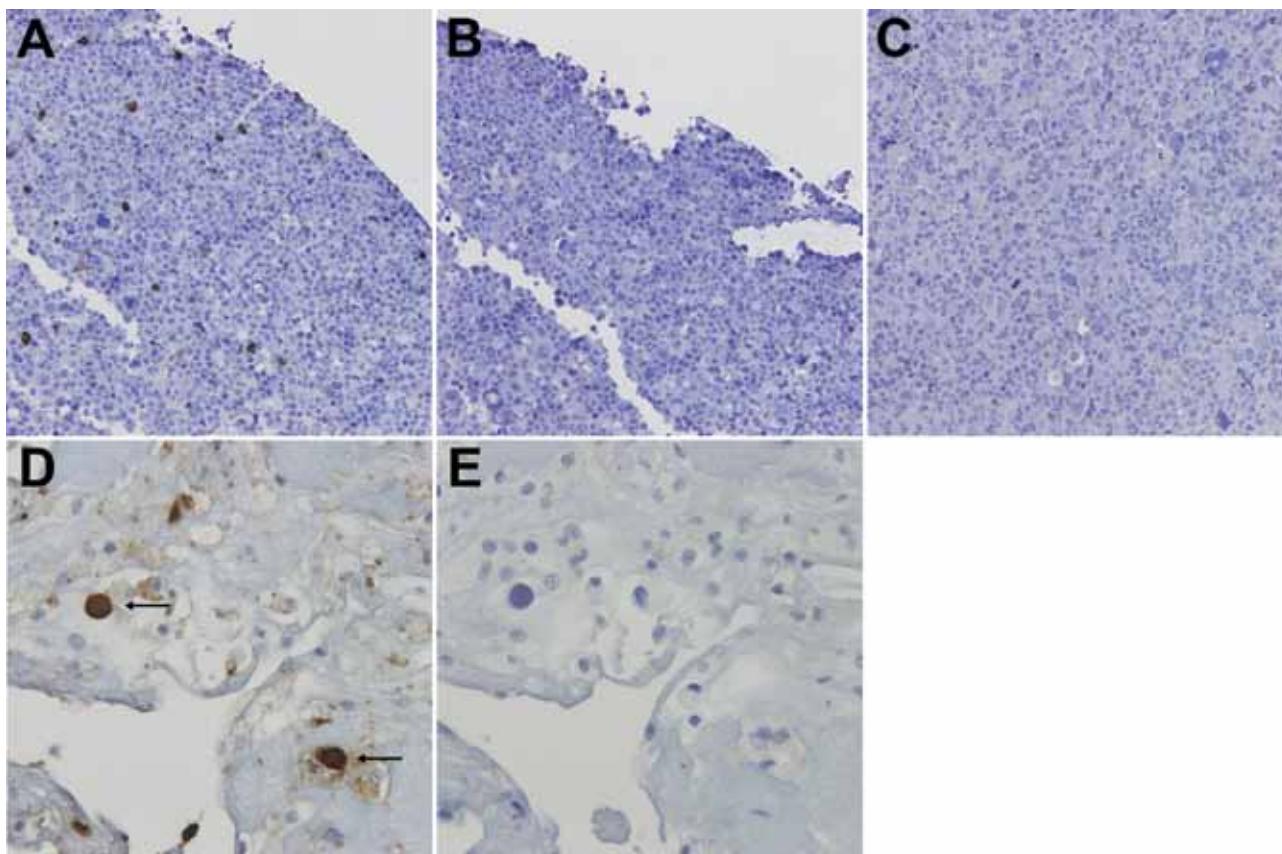


Figure 1. WU polyomavirus antigen in bronchoalveolar lavage specimens from lungs transplanted into a recipient (28-year-old woman) with Job syndrome. Immunohistochemical analysis of 293T cells transfected with pDEST26-WU–virus protein 1 and stained as follows. A) WU virus protein 1 monoclonal antibody (NN-Ab06). B) Isotype control. C) Mock transfected 293T cells stained with NN-Ab06. D) Bronchoalveolar lavage specimen stained with NN-Ab06 showing prominent dark staining of cells with enlarged nuclei and a ground glass appearance characteristic of viral cytopathic changes (arrows). E) Isotype control. Original magnifications ×400 in panels A–C and ×600 in panels D and E.

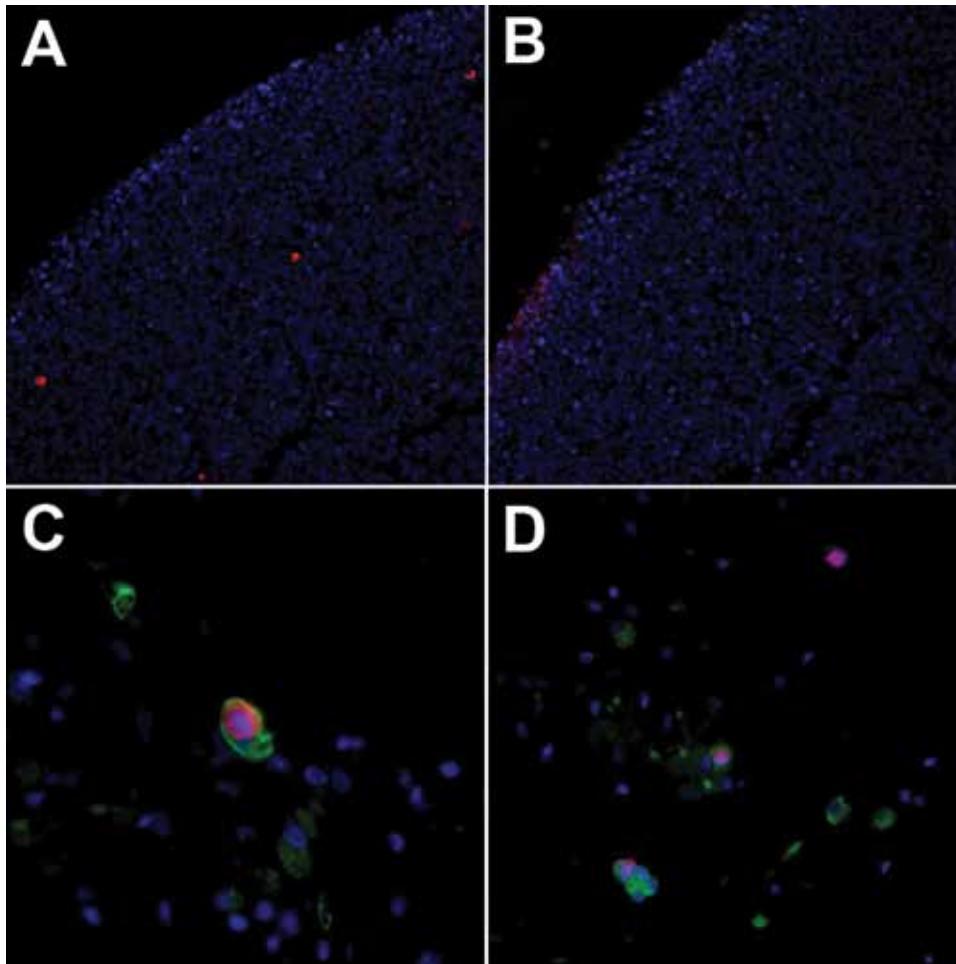


Figure 2. WU polyomavirus antigen in respiratory epithelial cells from lungs transplanted into a recipient (28-year-old woman) with Job syndrome. Immunofluorescence of 293T cells transfected with pDEST26-WU-virus protein 1 and stained with A) WU virus protein 1 polyclonal antibody (NN-Ab01) or B) preimmune serum. C) Double immunofluorescence with NN-Ab01 (red) and a monoclonal antibody against cytokeratin (green) showing a double-positive cell from the bronchoalveolar lavage specimen. D) Bronchoalveolar lavage specimen with multiple WU virus protein 1/cytokeratin double-positive cells. Original magnifications $\times 100$ in panels A and B, $\times 600$ in panel C, and $\times 400$ in panel D.

cells were observed (Figure 2, panel A); a serial section stained with preimmune serum at the same dilution showed a negative result (Figure 2, panel B).

For dIF, BAL sections were incubated first with the primary antibodies and then with fluorescently labeled secondary antibodies (#A10042 anti-rabbit-568 and #A10042 anti-mouse-488; Life Technologies). We observed cells positive for WU-VP1 and cytokeratin (Figure 2, panels C, D), which identified these cells as epithelial cells. Of the 136 WU-VP1-positive cells, 77 (57%) were also cytokeratin positive. A serial section of the BAL sample stained with an isotype-matched antibody to the cytokeratin antibody (#555746 mouse IgG1; BD Biosciences) and preimmune rabbit serum was negative.

We hypothesized that the remaining 43% of WU-VP1-positive, cytokeratin-negative cells might be macrophages. However, a dIF assay using NN-Ab01 and an antibody against CD68 (#M0814; Dako), a macrophage marker, showed WU-VP1 and CD68 single-positive cells but no double-positive cells. In addition, a stain with NN-Ab01 and an antibody against CD45 (#M351529-2;

Dako), a marker for hematopoietic cells, also showed negative results.

Conclusions

Before this study, to our knowledge, no specific cell type had been identified as susceptible to WUPyV infection. We found that WUPyV antigen was detected in human respiratory epithelial cells. The presence of nuclease-resistant viral DNA from the Optiprep gradient and detection of WU-VP1, which is believed to be expressed concomitantly with DNA replication (12), suggests that the cells were infected by WUPyV and that the WUPyV life cycle reached at least the stage of late gene expression. The clinical role of infection by WUPyV is uncertain, given that the patient was not experiencing any recognizable symptoms. Although we attempted to identify a second population of WU-VP1-positive, cytokeratin-negative cells, the etiologic features of these cells remains uncertain.

Our patient had Job syndrome, a primary immunodeficiency not previously associated with polyomavirus susceptibility. It is possible that immunosuppressant medications,

which include prednisone and tacrolimus, altered susceptibility to virus infection. Other human polyomaviruses are believed to exclusively cause disease in immunocompromised hosts. This case suggests that immunosuppression might also play a role in WUPyV infection and expands our understanding of WUPyV biology.

Acknowledgments

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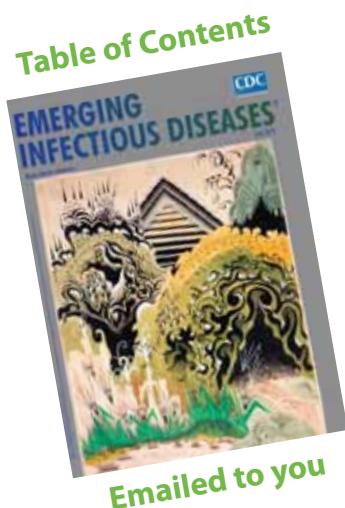
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Ms Siebrasse is a graduate student at Washington University, St. Louis, Missouri. Her research interests focus on discovery and characterization of novel polyomaviruses.

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Antibodies against *Borrelia burgdorferi* sensu lato among Adults, Germany, 2008–2011

Hendrik Wilking, Volker Fingerle, Christiane Klier, Michael Thamm, and Klaus Stark

To assess *Borrelia burgdorferi* sensu lato (the cause of Lyme borreliosis) seropositivity in Germany, we tested serum samples from health survey (2008–2011) participants. Seroprevalence was 5.8% among women and 13.0% among men; infection risk was highest among persons ≥ 60 years of age. Public health interventions, including education about risk factors and preventive measures, are needed.

Lyme borreliosis, the most common tickborne disease in the Northern Hemisphere, is caused by infection with spirochetes of the *Borrelia burgdorferi* sensu lato (s.l.) complex. Five genospecies are known to be pathogenic for humans: *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. bavariensis*, and *B. spielmanii* (1). In Europe, the bacterium is transmitted to humans through the bite of *Ixodes ricinus* ticks; in eastern Europe, *I. persulcatus* ticks can also transmit the bacterium.

In Europe, where the most common clinical manifestation of Lyme borreliosis is erythema migrans, followed by Lyme neuroborreliosis and Lyme arthritis (2), data are sparse regarding *B. burgdorferi* s.l. infection rates and risk factors (3). Persons of all ages are at risk for infection; however, surveillance data and prospective studies in Europe and the United States suggest that children and the elderly are particularly at risk (4–6). Population-based surveillance data suggest that Lyme borreliosis is endemic in eastern Germany: annual incidence is 20–35 cases/100,000 inhabitants (7). Regional differences in incidence are observed, but data cannot be easily compared because of reporting biases and differences in infection awareness.

The limited representativeness and comparability of Lyme borreliosis surveillance data are well documented (8). Under such conditions, population-based serosurveys with high representativeness can provide valid estimates of the force of infection (rate at which susceptible persons acquire Lyme borreliosis) and the lifetime risk for infection; however, seroprevalence estimates do not necessarily represent cases of clinical disease. In a population-based seroprevalence study among 1- to 17-year-old children in

Germany, seroprevalence increased cumulatively by age (9). We present data on the prevalence and determinants of *B. burgdorferi* s.l. seropositivity among adults in Germany during 2008–2011.

The Study

We estimated *B. burgdorferi* s.l. seroprevalence among participants of the German Health Interview and Examination Survey for Adults (DEGS). This nationwide cross-sectional survey assessed the health status of 18- to 79-year-old persons in Germany during 2008–2011 (10). The response rate was 48.4%; analysis of nonresponder questionnaires revealed high population representativeness. Data from standardized interviews were used to assess potential risk factors for seropositivity. Survey weights based on age, sex, residence in western or eastern Germany, and nationality (German vs. non-German) were calculated to correct for deviations from the German population statistics (December 31, 2010; <http://www.destatis.de>) and used throughout the analyses. The study was approved by the Ethical Review Board of the Medical School Charité, Berlin, Germany.

As recommended for serologic confirmation of clinical cases, serum samples were tested for the presence of *Borrelia burgdorferi* s.l. IgG. For screening, we used an ELISA based on *B. afzelii* extract antigen enriched with recombinant VlsE (an outer-surface protein) from *B. burgdorferi* s.s., *B. afzelii*, and *B. bavariensis*. ELISA-positive results were confirmed by line blot testing, which included purified antigens OspC, DbpA, and p83 from *B. afzelii*; recombinant VlsE from *B. burgdorferi* s.s. and *B. garinii*; and BmpA and DbpA from *B. garinii*, *B. bavariensis*, and *B. spielmanii*. Details regarding the tests are available in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/14-0009-Techapp1.pdf>). All tests were performed/interpreted according to the manufacturer's recommendations. To categorize samples by test results, we applied the rules shown in Figure 1.

We used sampling weights for all statistical analyses and accounted for the 2-stage sampling structure. Age-related prevalence was graphed and the values were smoothed by using the Lowess procedure of Stata 12.1 (StataCorp LP, College Station, TX, USA). We assessed differences between group prevalences (explanatory variables) by using the Wald test (univariable logistic regression) with 2-sided *p* values. Independent risk factors for seropositivity were investigated by using stepwise multivariable logistic regression. All plausible 2-way interactions were tested.

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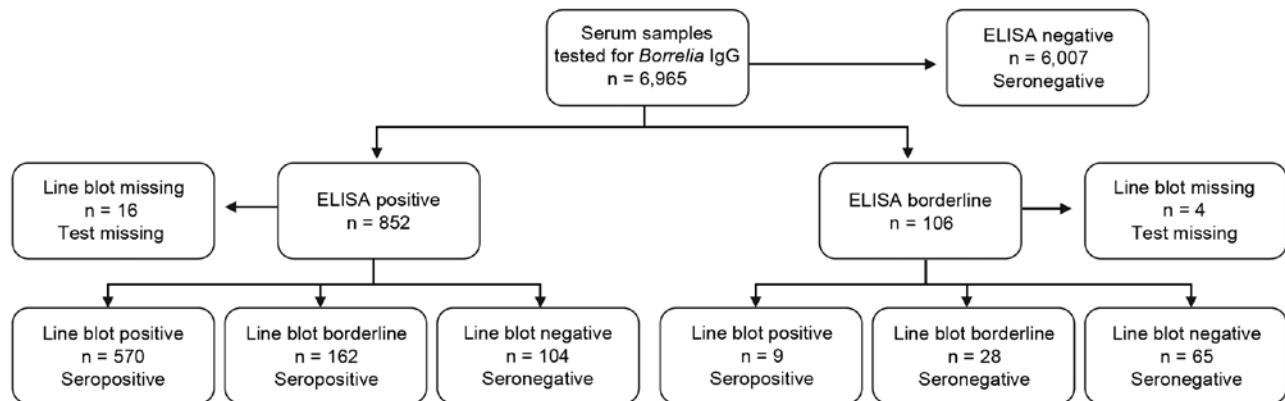


Figure 1. Categorization, according to ELISA and line blot test results, of serum samples tested for *Borrelia burgdorferi* sensu lato IgG, Germany, 2008–2011.

A total of 6,945 adults, representing 97.6% of the survey population with available blood samples, were included in the analysis. The overall weighted seroprevalence for *B. burgdorferi* s.l. was 9.4% (95% CI 8.4%–10.0%); seroprevalence was significantly higher among men (13.0%) than women (5.8%) ($p < 0.01$). Seroprevalence among both sexes increased by age (Figure 2); the increase was low among 18- to 50-year-old participants, most pronounced among participants >59 years of age, and higher among women than men >59 years of age. Seropositivity reached 20.0% (95% CI 16.9%–23.6%) in 70- to 79-year-old participants.

Among participants ≥ 18 years of age, more than twice as many men than women were seropositive for *B. burgdorferi* s.l. (odds ratio 2.44, 95% CI 2.01–2.96) (Table). No significant interaction between sex and age was found ($p = 0.075$). Independent risk factors for seropositivity were residence in a rural area ($p < 0.001$) and in southern Germany ($p = 0.032$). Non-German citizenship was negatively associated ($p = 0.004$) with seropositivity; having a dog/cat

in the house was not associated with a higher risk for seropositivity. To facilitate comparison of our data with data from serosurveys lacking confirmatory testing, we have made our ELISA results available online (online Technical Appendix Table).

Conclusions

B. burgdorferi s.l. infections are common in Germany; Lyme borreliosis is endemic in all regions, but case numbers are highest in southern Germany. Previously identified risk factors for *B. burgdorferi* s.l. seropositivity in children (male sex and living in rural areas, small-sized towns, or southern Germany) were identified as risk factors for seropositivity among adults in our study. Holding a cat was previously shown to be a risk factor for children/adolescents (9), but was not a risk factor in our study. Seroprevalence among the oldest age group indicates that at least one fifth of the German population becomes infected with *B. burgdorferi* s.l. during their lifetime.

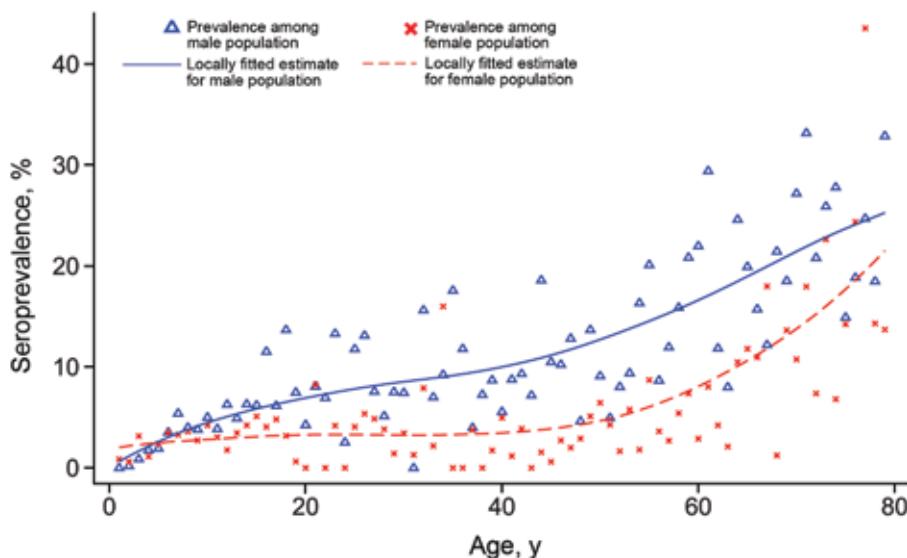


Figure 2. Estimated seroprevalence of *Borrelia burgdorferi* sensu lato IgG among the male and female population, Germany, 2008–2011. For comparison, results of Dehnert et al. (9), a previous study among children/adolescents <18 years of age, were added to the graph.

Table. Stratified seroprevalence of *Borrelia burgdorferi* sensu lato IgG detected by combined ELISA and line blot testing in adults and results of weighted logistic regression analysis of potential risk factors for seropositivity, Germany, 2008–2011*

Characteristic†	No. positive/ no. total†	Prevalence (95% CI)	Univariable analysis		Multivariable analysis	
			OR (95% CI)	p value	OR (95% CI)	p value
Sex						
F	240/3,614	5.8 (4.9–6.7)	Ref	Ref	Ref	Ref
M	501/3,331	13.0 (11.4–14.8)	2.44 (2.01–2.96)	<0.001	2.61 (2.15–3.16)	<0.001
Age group, y						
18–29	62/1,043	6.0 (4.5–8.0)	Ref	Ref	Ref	Ref
30–39	50/829	6.3 (4.4–9.0)	1.05 (0.64–1.69)	0.854	1.07 (0.67–1.72)	0.779
40–49	83/1,263	6.4 (5.0–8.2)	1.07 (0.72–1.58)	0.737	1.04 (0.69–1.55)	0.856
50–59	126/1,373	8.5 (6.8–10.7)	1.46 (1.01–2.10)	0.043	1.39 (0.97–1.99)	0.069
60–69	186/1,361	13.2 (10.9–15.9)	2.37 (1.65–3.40)	<0.001	2.37 (1.65–3.45)	<0.001
70–79	234/1,076	20.0 (16.9–23.6)	3.91 (2.77–5.51)	<0.001	4.02 (2.84–5.70)	<0.001
Residence location						
West‡	484/4,748	9.1 (8.0–10.4)	Ref	Ref	–	–
East§	257/2,197	10.4 (8.5–12.6)	1.15 (0.89–1.49)	0.273	–	–
North¶	181/1,767	9.0 (7.2–11.0)	1.11 (0.82–1.51)	0.479	1.16 (0.86–1.57)	0.318
Middle#	304/3,087	8.1 (6.7–9.8)	Ref	Ref	Ref	Ref
South**	256/2,091	11.2 (9.4–13.3)	1.43 (1.08–1.88)	0.011	1.34 (1.03–1.75)	0.032
Population of residence municipality						
<5,000	189/1,258	15.4 (12.8–18.4)	2.50 (1.85–3.30)	<0.001	2.13 (1.54–2.97)	<0.001
5,000 to <20,000	185/1,685	10.0 (8.0–12.5)	1.51 (1.11–2.07)	0.010	1.33 (0.96–1.84)	0.082
20,000 to <100,000	193/2,030	8.4 (6.9–10.2)	1.24 (0.92–1.66)	0.154	1.21 (0.88–1.67)	0.231
>100,000	174/1,972	6.9 (5.6–8.4)	Ref	Ref	Ref	Ref
Foreign national††						
No	721/6,528	10.0 (8.9–11.2)	Ref	Ref	Ref	Ref
Yes	18/396	4.3 (2.5–7.5)	0.41 (0.22–0.75)	0.004	0.54 (0.30–0.90)	0.041
Pet in household						
None	502/4,596	9.5 (8.4–10.7)	Ref	Ref	–	–
Any	217/2,182	9.3 (7.8–11.0)	0.98 (0.80–1.20)	0.834	–	–
Dog						
No	639/5,909	9.4 (8.4–10.6)	Ref	Ref	–	–
Yes	80/858	9.3 (7.0–12.2)	0.98 (0.71–1.35)	0.909	–	–
Cat						
No	622/5,886	9.2 (8.2–10.3)	Ref	Ref	–	–
Yes	119/1,077	10.3 (8.1–13.0)	1.13 (0.87–1.47)	0.356	–	–
Other animals						
No	655/6,001	9.7 (8.8–10.6)	Ref	Ref	–	–
Yes	64/766	7.7 (5.8–10.1)	0.78 (0.56–1.07)	0.127	–	–
Total	741/6,945	9.4 (8.4–10.0)	–	–	–	–

*OR, odds ratio; Ref, reference; –, not included in the final model.

†Unweighted.

‡Western states: Baden-Württemberg, Bavaria, Bremen, Hamburg, Hesse, Lower Saxony, Northrhine-Westfalia, Rhineland-Palatinate, Saarland, Schleswig-Holstein.

§Eastern states: Berlin, Brandenburg, Mecklenburg–West Pomerania, Saxony, Saxony-Anhalt, Thuringia.

¶Northern states: Schleswig-Holstein, Hamburg, Lower Saxony, Bremen, Berlin, Brandenburg, Mecklenburg-West Pomerania.

#Middle states: Northrhine-Westfalia, Hesse, Saxony, Saxony-Anhalt, Thuringia.

**Southern states: Rhineland-Palatinate, Baden-Württemberg, Bavaria, Saarland.

††Defined as persons holding a foreign citizenship.

B. burgdorferi s.l. IgG seroprevalence among blood donors in Italy (4.9%; n = 365) (11) and Romania (4.3%; n = 1,598) (12) was lower than the seroprevalence in our study. Prevalences higher than those in our study have been shown in serosurveys in areas of high disease endemicity in southwestern Germany (16.9%; n = 1,228) (13) and Finland (19.3%; n = 3,248) (14). In serosurveys of persons with high exposure to ticks (e.g., forestry and agricultural workers), similar or higher seroprevalence rates have been described.

Seroprevalence rates among men in our study were strikingly higher than rates among women, indicating that tick contact/spirochete transmission is more frequent

among men. Prospective studies in Germany and Sweden and surveillance data from Germany show no differences in clinical cases (except only a slight preponderance among women) that would point to substantial sex-specific differences in the development of clinical disease (5–7).

The age distribution for seropositivity reflects the population's cumulative exposure to *B. burgdorferi* s.l.. An increased risk for infection among children and persons >59 years of age suggests that leisure activities/behaviors rather than occupational exposure are the main risk factor for infection. Alternatively, these findings might be explained by a birth-cohort effect, in which the force of infection was lower during 1950–1990.

Persons living in urbanized areas had a lower probability for *B. burgdorferi* s.l. seropositivity, suggesting that exposure to infected ticks is higher in rural areas. However, urban populations are also at substantial risk for infection. Seropositivity is not equivalent to clinical disease; thus, seropositivity rates among the different population groups may not necessarily reflect the true effect of infection on disease burden. Furthermore, a US study showed that persons can be consecutively infected by different *B. burgdorferi* strains and experience clinical manifestations with each infection (15).

Our seroprevalence estimates can be used, within the context of clinical diagnoses, to assess the likelihood of Lyme borreliosis in persons with test results positive for *B. burgdorferi* s.l. IgG. To reduce the incidence and disease burden of Lyme borreliosis, enhanced public health interventions are needed, including education campaigns targeted to parents, children, and the elderly about potential risk factors and preventive measures for Lyme borreliosis.

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Foot-and-Mouth Disease Virus Serotype SAT 3 in Long-Horned Ankole Calf, Uganda

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After a 16-year interval, foot-and-mouth disease virus serotype SAT 3 was isolated in 2013 from an apparently healthy long-horned Ankole calf that grazed close to buffalo in Uganda. The emergent virus strain is $\approx 20\%$ different in nucleotide sequence (encoding VP1 [viral protein 1]) from its closest relatives isolated previously from buffalo in Uganda.

Foot-and-mouth disease (FMD) remains one of the most economically important diseases of livestock, costing \approx US \$10 billion annually (1). Outbreaks occur in many countries, and normally disease-free countries can incur huge costs after incursions (e.g., the United Kingdom in 2001). The disease results from infection with FMD virus (FMDV, the prototypic aphthovirus within the *Picornaviridae* family) (2). Seven serotypes of FMDV are known; serotypes O and A are widely distributed, and the Southern African Territories (SAT) serotypes (1, 2, and 3) usually are restricted to Africa. Serotype Asia 1 has never circulated within Africa; serotype C has not been identified anywhere since 2005 (2,3). SAT 3 FMDV is the least well-characterized serotype; the most recent incidence of SAT 3 reported by the FMD World Reference Laboratory (Pirbright Institute, Woking, UK) was in buffalo within the Kruger National Park (South Africa) in 2006. In contrast, SAT 1 and SAT 2 FMDVs are much more common; a major incursion of SAT 2 into the Middle East occurred in 2012 (4), and outbreaks caused by these serotypes have occurred in many African countries (http://www.wrlfmd.org/fmd_genotyping/2013.htm).

In Uganda, FMD is endemic, and serotypes O and SAT 2 are the most common. In Uganda, SAT 3 FMDV was most recently identified in 1997 in buffalo in the Queen

Elizabeth National Park (QENP) (5). SAT 1 and SAT 2 viruses were isolated from buffalo in QENP in 2006, and serologic test results indicated the presence of antibodies against SAT 3 virus; however, because cross-reactivity between serotypes occurs in these assays, this finding was not conclusive (6).

The Study

In 2013, as part of a study of FMDV transmission between wildlife, especially African buffalo (*Syncerus caffer*), and domestic animals, 20 long-horned Ankole cattle (≈ 6 months of age) were introduced as sentinel animals into Nyakatonzi (Kasese District), in close proximity to the QENP. At the time they were transported, these animals, originating from another area where FMD outbreaks had not been reported for ≈ 10 years, had no circulating antibodies against FMDV nonstructural proteins (NSPs) (measured by using the PrioCHECK FMDV NS ELISA kit [Prionics, Schlieren-Zurich, Switzerland]). Blood and probang samples (comprising oropharyngeal scrapings and fluid) were obtained from individual animals at 2-week intervals after their entry to the farm from which they moved regularly into the QENP for pasture and water. These sentinel animals freely mixed and grazed with buffalo (observed within a few meters of each other) and other local cattle. More than 6,000 buffalo are present within the QENP.

No clinical signs of FMD were observed in the sentinel cattle, but serum samples were assayed for antibodies against FMDV NSPs (serotype independent); RNA was extracted from the probang samples and analyzed for FMDV genomes by using pan-serotypic real-time reverse transcription PCR (RT-qPCR) (7). FMDV RNA was clearly detected (cycle threshold 21) in the probang sample from animal no. 34 at 2 weeks after its introduction into the QENP. Antibodies against FMDV NSPs had developed in this animal (Table 1), and we detected high-titer antibodies against both FMDV SAT 1 and SAT 3 antigens using solid-phase blocking ELISAs (SPBEs) (Table 1); hence this calf simultaneously had FMDV RNA in the oropharynx and antibodies against FMDV in serum. FMDV is maintained in the oropharynx of cattle for ≈ 10 days after infection (8) and continues after viremia has resolved coincident with the production of antibodies against FMDV. Infectious FMDV (albeit at low levels) can be maintained within the oropharynx after infection for up to 3 and 5 years in “carrier” cattle and buffalo, respectively (9). Seroconversion against FMDV NSPs was observed in a second animal (no. 33) by 30 days after introduction to the QENP (Table 1).

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Table 1. Detection of anti-FMDV in serum and FMDV RNA in probang samples from long-horned Ankole calves, Uganda, 2013*

Sampling day†	Calf no. 34						Calf no. 33					
	Anti-NSP, PI	SPBE titers‡			FMDV RNA in probang, C _t	Anti-NSP, PI	SPBE titers‡			FMDV RNA in probang, C _t		
	O	SAT 1	SAT 2	SAT 3		O	SAT 1	SAT 2	SAT 3			
0	22	–	–	–	–	35	–	–	–	–		
14	55	–	160	–	80	25	<10	10	10	10		
30	72	–	80	–	40	63	20	160	10	10		

*C_t, cycle threshold; ELISA, enzyme-linked immunosorbant assay; FMDV, foot-and-mouth disease virus; PI, percentage inhibition values in the NSP ELISA (values >50% are considered positive and are indicated in boldface); NSP, nonstructural protein; RT-qPCR, real-time reverse transcription PCR; SAT, Southern African Territories; SPBE, solid-phase blocking ELISA; –, negative sample.

†Day 0 was defined as the day the sentinel animals arrived at the farm in Nyakatonzi (Kasese District).

‡An in-house serotype-specific SPBE was used; samples positive at 1:10 dilution were titrated; values are reciprocals of highest positive dilution.

§FMDV RNA in probang samples was assayed by RT-qPCR, and virus isolation was attempted from probang samples with detectable RNA and some other selected samples. The isolation of SAT 3 FMDV from the RT-qPCR–positive probang sample from calf no. 34 was demonstrated by serotype-specific antigen ELISA and confirmed by sequencing.

In the SPBEs, reactivity against SAT 1 was mainly detected in this animal. However, during the early stage of seroconversion, cross-reactivity occurs between the serotypes in these assays (K. Tjørnehøj, unpub. data).

An aliquot of the probang sample from calf no. 34 was inoculated onto primary bovine thyroid cells for virus isolation. We observed cytopathic effects within 48 hours and assayed the virus harvest using serotype-specific antigen ELISAs. We observed a strong signal (optical density 1.385), indicating SAT 3 FMDV with no significant signal for other serotypes (data not shown). The presence of FMDV RNA in the cell harvest was demonstrated by using RT-qPCR (cycle threshold 11), indicating isolation and growth of FMDV; the isolate was named SAT 3 UGA/1/13. To characterize this strain, we amplified the RNA region encoding viral protein (VP) 1 by RT-PCR; the amplicon

(821 bp) was sequenced with a BigDye Terminator v. 3.1 Cycle Sequencing Kit and an ABI PRISM 3730–DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Using BLAST (<http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi>) in MEGA5 (10), we found the sequence to be most closely related (81% identity) to the Ugandan SAT 3 buffalo isolate (UGA/2/97) (5) (Figure 1, panel A). The range of divergence was 19%–36% in this region of the genome (published VP1 coding sequences are mainly incomplete [5,11–13]; thus we analyzed only 390 nt from the 648 nt encoding VP1). The UGA/1/13 strain is most closely related to earlier Uganda SAT 3 viruses but is within a different lineage and is more divergent from SAT 3 viruses from southern Africa. The UGA/27/70 and UGA/2/97 viruses were assigned to topotypes V and VI, respectively (5); because the UGA/1/13 is ≈20% different from these

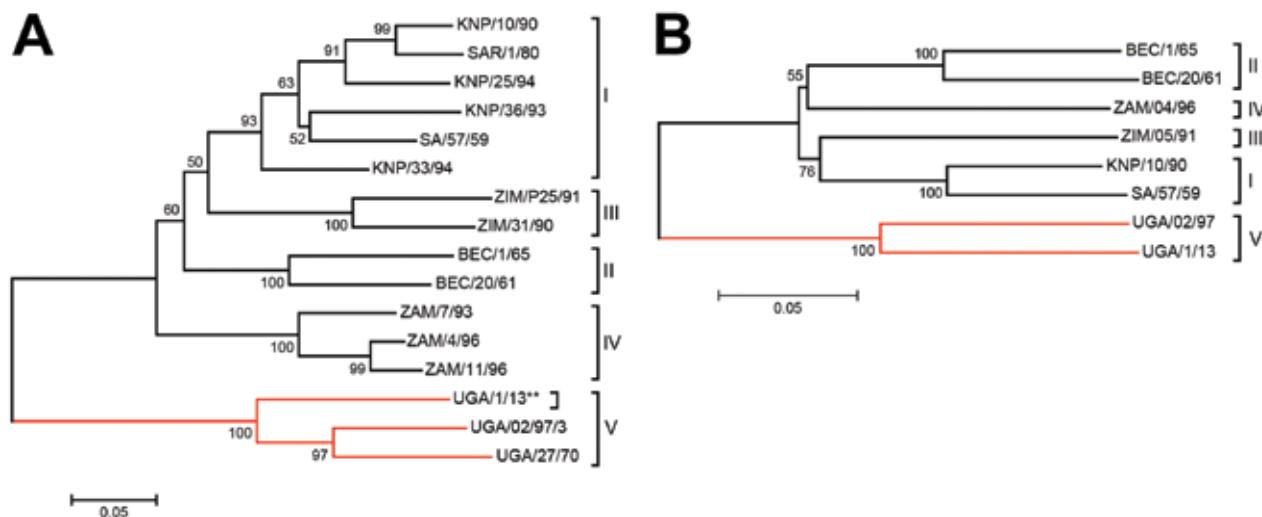


Figure 1. Neighbor-joining trees showing the relationships between A) the partial VP1 coding sequences (390 nt) and B) the complete P1 capsid protein coding sequence (2223 nt) from the SAT 3 FMDV UGA/1/13 isolate (marked with **) and other SAT 3 FMDVs within the indicated topotypes defined previously (5; http://www.wrlfmd.org/fmdv_seqs/fmdv-sat3_seq.aspx). The branches containing the Uganda viruses are indicated by the red lines. Sequences, other than for UGA/1/13, were obtained from GenBank and have been published previously (5,11–13). Bootstrap values are indicated. BEC, Bechuanaland (former name for Botswana); FMDV, foot-and-mouth disease virus; KNP, Kruger National Park (in South Africa); P1, precursor for the 4 capsid proteins; SAT, Southern African Territories; SAR, Republic of South Africa; UGA, Uganda; VP, viral protein; ZAM, Zambia; ZIM, Zimbabwe; I–V, topotypes. Scale bars indicate nucleotide substitutions per site.

	10	20	30	40	50	60
BEC/1/65	TTSAGEGADV	VTTDVTTHGG	EVSVPRRQHT	NVEFLLDRFT	HVGKVNESRT	ISLMDTKEHT
BEC/20/61I.TI.GH..	.C.L.....
KNP/10/90G..	A.DT.....I.TITG.K.	.D.....
SA/57/59G..	V.DT.....I.TITA.K.	.D.....
UGA/02/97	...S.....	.S.T.E....	TEQ.A.....	D.A.....	.I.SMAGTK.	LD.LT.....
UGA/1/13S.T.E....	TAQ.A.....	D.A.....	.I.P.TG.K.	LD.LT.....
ZAM/04/96N.TVH.....I.AMTT.K.	...L.....
ZIM/05/91	T.DT.....I.SITA.K.	.D.LE.....
	70	80	90	100	110	120
BEC/1/65	LVGAILRSAT	YYFCDLEVAI	LGTAPWAAWV	PNGCPHTGRV	EDNPVVHSKG	SVVRFALPYT
BEC/20/61V	.N.KY....D..
KNP/10/90V	.GK.V...T..A..	G.T.....
SA/57/59V	.SEK.T...E..A.N	G.A.....
UGA/02/97V	K.DS.Y....EVS..FA..	GLA.....
UGA/1/13	M.....V	.D.EH...IEVNNIFA..	G.A.....
ZAM/04/96V	.D.E.V...D..N	G.T.....F.
ZIM/05/91	...L.....V	.N.K.VG..D..A..	N.T.....
	130	140	150	160	170	180
BEC/1/65	APHGVLATVY	NGNCKYSETQ	RVTSRRGDLA	VLAQRVENET	TRCLPTTFNF	GRLLCCEEGDA
BEC/20/61T..	.AP.....G	A.SR.....	...I.....S..V
KNP/10/90K..	H.VP.....R.....DT..V
SA/57/59	...R.....K..	H.VA...M.S.I	...R.....T.EV
UGA/02/97	...Q.....	.T.V.RKNA	P..P.....Q	A.QA..DA.R	E..I..S...	...YA.S.EL
UGA/1/13	...R...I.	.T.V.KKNA	P.....Q	.Q...DA.R	E..I..S...	...YV.S.NL
ZAM/04/96S...	.T...K..	...P.....	.ST...T.Q	E.....A...DS..V
ZIM/05/91T.	.T...K..	S.KP...M.G.Q	Q..K.....DS..V
	190	200	210	216		
BEC/1/65	YYRMKRAELY	CPRPLRVRYT	HTTDRYKTPL	VKPKDKQ		
BEC/20/61T...A.....	...E..		
KNP/10/90A...AK.	.A....		
SA/57/59IK.	.A....		
UGA/02/97	.L.....	...WIF....V..	T..E..		
UGA/1/13	.L.....	...W.F....H.VS.	...E..		
ZAM/04/96VA.	...E..		
ZIM/05/91M....VA.	.S.A..		

Figure 2. Predicted VP1 aa sequences of the 8 SAT 3 FMDVs used in the phylogenetic comparison in Figure 1, panel B. Clear similarities between the UGA/02/97 and UGA/1/13 viruses are apparent. BEC, Bechuanaland (former name for Botswana); FMDV, foot-and-mouth disease virus; KNP, Kruger National Park (in South Africa); SAR, Republic of South Africa; SAT, Southern African Territories; UGA, Uganda; VP, viral protein; ZAM, Zambia; ZIM, Zimbabwe.

strains, it could be designated as a new topotype. However, it seems better to classify these Uganda SAT 3 strains within a single topotype (V). The genome sequence (8,268 nt) of this SAT 3 virus was determined (GenBank accession no. KJ820999) from 17 overlapping amplicons (primer sequences available on request). Only 3 other full-genome sequences for SAT 3 FMDVs have been published (13); these are from southern African isolates obtained \approx 50 years ago. These sequences are \approx 80% identical to the UGA/1/13 isolate; this degree of diversity is not unexpected considering

the geographic and temporal separation between them. Relationships between the SAT 3 viruses were examined for different regions of the genome (Table 2). The P1–2A capsid coding regions differ the most (\approx 27%). Phylogenetic relationships between known SAT 3 P1 coding sequences were determined (Figure 1, panel B). Alignment of the predicted amino acid sequences for VP1 (Figure 2) indicates high levels of variation within important antigenic regions of the virus (especially the G–H loop, residues 140–160). Neighbor-joining trees for other regions of the genome showed very

Table 2. Comparison of full-genome nucleotide sequences between different regions of the SAT 3 UGA/1/13 isolate and other SAT 3 FMDVs, 2013*

Sequence region	Length, nt	Nucleotide differences from SAT 3 UGA/1/13, %†		
		SA/57/59	BEC/20/61	BEC/1/65
5' UTR‡ + L	1,680	24.0	24.1	22.7
P1–2A	2,282	26.6	27.1	27.1
P2	1,464	13.7	14.2	14.6
P3	2,700	13.8	13.8	13.9
3' UTR§	118	5.9	8.4	5.0
Full genome	8,268	19.1	19.5	19.3

*BEC, Bechuanaland (former name for Botswana); FMDV, foot-and-mouth disease virus; L, leader protein coding sequence; SAT, Southern African Territories; UGA, Uganda; UTR, untranslated region.

†These sequences were previously published (13).

‡The region from the poly(C) tract to the first initiation codon was used for the comparison.

§Excluding the poly(A) tail.

similar relationships (data not shown), indicating that these viruses are monophyletic.

Conclusions

Approximately 16 years after the most recent isolation of a SAT 3 FMDV from buffalo in Uganda, a new isolate (UGA/1/13) was obtained from an apparently healthy long-horned Ankole calf that was newly introduced into the QENP. To our knowledge, this is the first isolation of SAT 3 FMDV from cattle in East Africa. The VP1 coding sequence was ≈20% different from the most closely related virus strains within Uganda and up to 36% divergent from SAT 3 viruses from southern Africa. The Ugandan SAT 3 viruses should be classified within a single topotype (V), but this requires modification of the topotype definition used previously (5). Studies are needed to determine the consequences of infection of intensively farmed cattle by this virus.

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The work presented here is part of a study clarifying the role played by cattle in maintaining the spread of FMDV in Uganda.

Dr. Dhikusooka is a veterinarian undertaking a PhD at Makerere University, Uganda, in a collaborative research program with the DTU National Veterinary Institute, Denmark. His research interests include epidemiology and dynamics of disease in livestock.

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Continuing Effectiveness of Serogroup A Meningococcal Conjugate Vaccine, Chad, 2013

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In 2011, vaccination with a serogroup A meningococcal polysaccharide conjugate vaccine was implemented in 3 of 23 regions in Chad. Cases of meningitis declined dramatically in vaccinated areas, but an epidemic continued in the rest of Chad. In 2012, the remaining Chad population was vaccinated, and the epidemic was halted.

For >100 years, countries in the meningitis belt of Africa have experienced intermittent epidemics of meningococcal meningitis, caused mainly by the serogroup A meningococcus (1). After development and prequalification of a new serogroup A meningococcal polysaccharide/tetanus toxoid conjugate vaccine (PsA-TT) in 2009 (2), vaccination with PsA-TT across the meningitis belt commenced in 2010, starting with persons 1–29 years of age in Burkina Faso and parts of Mali and Niger (3). Little transmission of the serogroup A meningococcus was occurring in these countries at the time of vaccine introduction, making evaluation of its effectiveness difficult.

In contrast, in Chad, PsA-TT was introduced in the middle of a serogroup A meningococcal epidemic, and vaccination with PsA-TT commenced at the end of 2011, shortly before the 2012 epidemic season. At this time, vaccination of persons 1–29 years of age (target 1.8 million) was undertaken in the capital N'Djamena, Mayo Kebbi Est, and Chari Baguirmi (4), designated here as the N'Djamena regions (Figure 1). In 2012, the vaccination program was

extended to the rest of the country (target 5.9 million) (Figure 1). During the 2012 meningitis season, the incidence of meningitis decreased by >90% in vaccinated areas compared with the rest of the country, and a similar reduction in the incidence of carriage of serogroup A *Neisseria meningitidis* was found, as reported previously (4). We report on the incidence of meningitis during the 2013 meningitis season after vaccination of persons 1–29 years of age in areas with no prior vaccination program.

The Study

In Chad, health districts provide aggregated weekly data on meningitis and other notifiable diseases to the Ministry of Health. This system was reinforced in 2012 and 2013 by case-based surveillance supported by the Centre du Support en Santé Internationale in the N'Djamena regions, and also by Médecins sans Frontières in Moissala, a district ≈800 km from NDjamena (Figure 1). Cerebrospinal fluid (CSF) specimens obtained from persons with suspected cases of meningitis were transported to the national reference laboratory in NDjamena. Isolated strains of *N. meningitidis* were sent to the World Health Organization (WHO) Intercountry Support Team, Ouagadougou, Burkina Faso, and to the WHO Collaborating Centre for Reference and Research on Meningococci in Oslo. Information about the laboratory methods used to isolate and characterize meningococci is provided elsewhere (4). Data from the 2009 census were used to calculate incidence rates. We used a negative binomial regression model to assess the effect of PsA-TT on the incidence of meningitis in the N'Djamena regions in 2012 and in the whole country in 2013; we used weekly data obtained during the epidemic period (weeks 1–26) during 2009–2013.

The incidence of meningitis in Chad during 2009–2013 and its association with the introduction of PsA-TT are shown in Figure 2. During weeks 1–26 of 2012, the incidence of reported meningitis among persons in all age groups in the N'Djamena regions that received vaccine was 2.5 cases/100,000 population (57/2.3 million); during the previous year, incidence was 31.8/100,000 (732/2.3 million). Meningitis incidence remained low in the N'Djamena regions in 2013 at 1.1/100,000 (25/2.3 million). In the rest of the country, in which vaccination was implemented during 2012 only, meningitis incidence decreased from 43.8/100,000 (3,809/8.7 million) in weeks 1–26 of 2012 to 2.8/100,000 (247/8.7 million) during the same period in 2013, a 96% reduction ($p<0.0001$). The incidence rate

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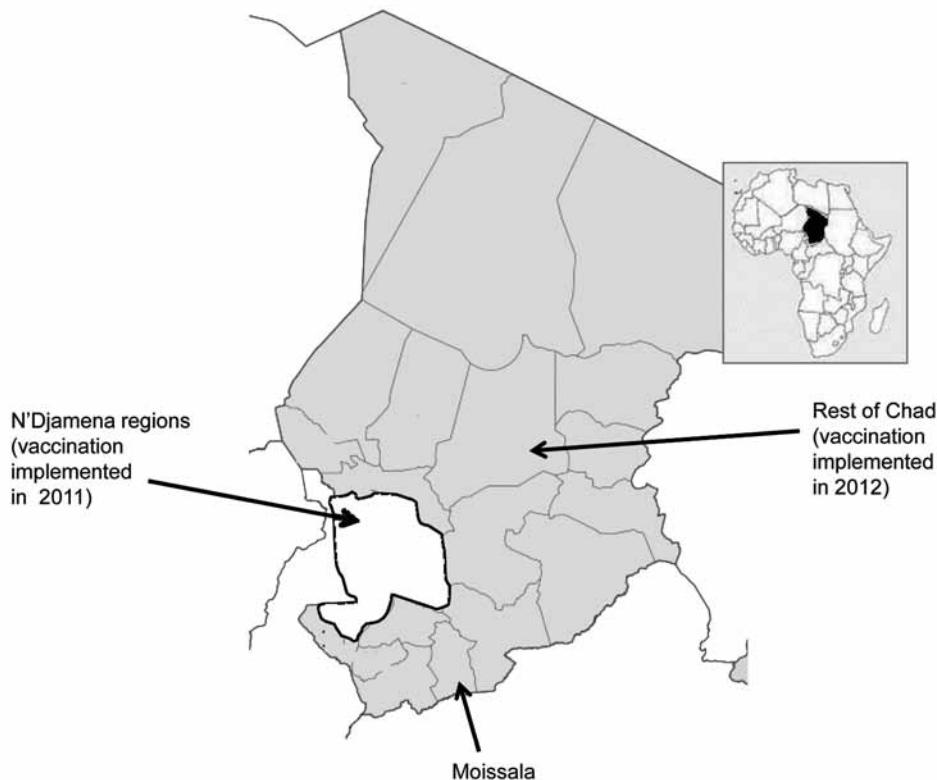


Figure 1. Areas of Chad in which vaccination with serogroup A meningococcal polysaccharide/tetanus toxoid conjugate vaccine was implemented in 2011 (white) and 2012 (gray). Inset shows location of Chad in Africa.

ratio for vaccinated versus unvaccinated populations was estimated by using data across the whole study period with a negative binomial regression model; the incidence rate ratio was 0.104 (95% CI 0.052–0.207).

Fewer CSF specimens were submitted to the national reference laboratory in N'Djamena in 2013 than in 2012 (Table), but the proportion of reported cases for which CSF samples were submitted increased from 8.3% (273/3,308) in 2010, 7.9% (516/6,540) in 2011, and 9.5% (366/3,866) in 2012, to 39.3% (106/272) in 2013 (Pearson χ^2 $p < 0.0001$). The proportion of reported cases for which CSF samples were submitted from the N'Djamena regions was highest in 2010, when the main pediatric unit for N'Djamena was in the same hospital as the national reference laboratory (the unit moved to another hospital in 2011) and in 2012, when case-based surveillance was introduced. In Moissala, the proportion of cases for which CSF samples were submitted increased from 22% (74/341) in 2012 to 119% (56/47) in 2013, the latter figure being attributed to undernotification. During weeks 1–26 of 2013, a total of 106 CSF specimens were received by the national reference laboratory; 13 yielded *Streptococcus pneumoniae*, 4 *Haemophilus influenzae* type b, 2 *N. meningitidis* serogroup W, and 1 (obtained from a 3-year-old child who had not received PsA-TT) serogroup A *N. meningitidis*. Four infections were

caused by other pathogens. This finding differed markedly from those of previous years (2010–2012), when the predominant organism was *N. meningitidis* serogroup A, and only a few cases caused by *N. meningitidis* serogroup W and *S. pneumoniae* were also identified. The predominance of serogroup A infection in Chad during 2010–2012 was confirmed among the CSF specimens examined at the National Institute of Public Health in Oslo. All fully characterized serogroup A strains were porA 20.9, FetA F3.1, sequence type (ST) 7 (ST5 complex), and all serogroup W strains were porA 5.2, FetA F1–1, ST11 (ST11 complex). Although national reference laboratory data were not available in Chad for 2009, the predominant organism identified from CSF specimens received at the National Institute of Public Health in Oslo in 2009 was *N. meningitidis* serogroup W (11/14 serogrouped strains), also porA 5.2, FetA F1–1, and ST11 (ST11 complex) (5).

Conclusions

We previously reported a >90% reduction in incidence of meningitis among vaccinated populations in Chad in 2011 (4). Here we report a similar reduction in the incidence of meningitis in 2013 from that in 2012 for populations vaccinated only during the second year of the vaccination campaign. The epidemic curve (Figure 2) suggests that by 2013,

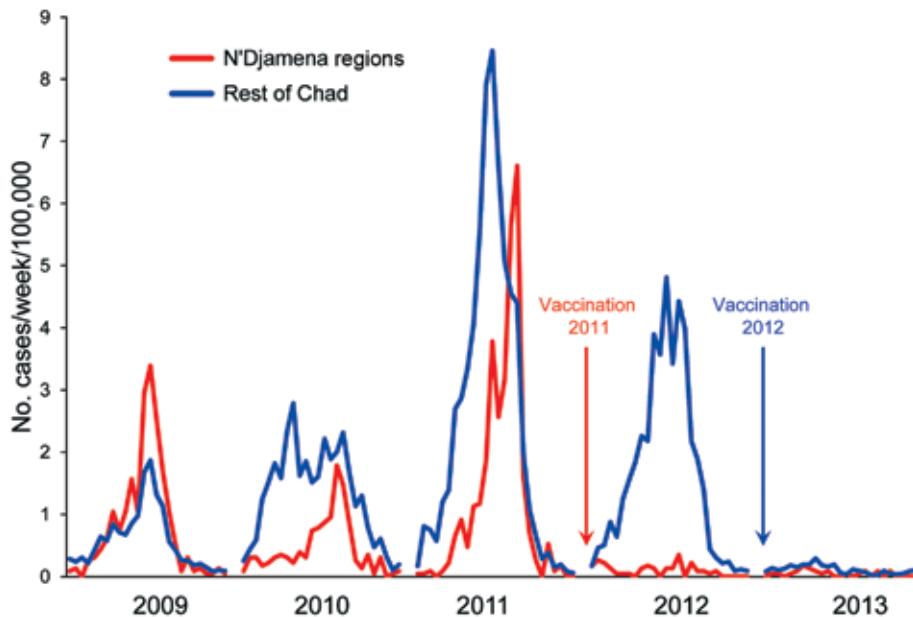


Figure 2. Incidence (no. cases/100,000 population) during weeks 1–26 of reported cases of meningitis in regions of Chad where persons 1–29 years of age were vaccinated with serogroup A meningococcal polysaccharide/tetanus toxoid conjugate vaccine at the end of 2011 and in 2012.

the *N. meningitidis* serogroup A epidemic in Chad was waning and that fewer cases of serogroup A meningitis would have occurred during 2013 than during 2012, even in the absence of vaccination. However, the incidence of meningitis dropped lower in 2013 than would have been expected as a result of a natural decline, and only 1 serogroup A isolate was obtained at the national reference laboratory despite improved CSF sampling. This finding provides strong additional evidence of vaccine effectiveness for preventing serogroup A meningococcal disease in Chad.

If the effectiveness of PsA-TT vaccination seen in Chad and Burkina Faso (6,7) is replicated across the meningitis belt of Africa and if vaccine coverage can be sustained through introduction of PsA-TT into the infant immunization program and/or through mass campaigns, serogroup A epidemics could disappear from the meningitis belt. However, past experience has shown that meningococci

belonging to serogroups C, W, or X can cause substantial epidemics (8–10); therefore, continuing surveillance will be needed to determine how the epidemiology of meningococcal disease in the meningitis belt of Africa is changed by the successful introduction of PsA-TT (11). The Chad Ministry of Health has approved a plan to support and develop case-based surveillance in the N'Djamena regions, Moissala, and 3 other selected health districts.

Acknowledgments

We acknowledge the major contributions of the Meningitis Vaccine Project and their colleagues in the control of epidemic meningitis in Africa through the development of PsA-TT.

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Table. Diagnoses of suspected meningitis cases from CSF specimens, Chad, weeks 1–26, 2010–2013*

Location, year	No. reports of suspected meningitis	CSF specimens, no. (%)	Diagnosis†						
			<i>N. meningitidis</i>				<i>S. pneumoniae</i>	<i>H. influenzae</i> type b	Other
			A	W	X	Other			
N'Djamena regions									
2010	268	158 (58.9)	27	2	0	2	8	0	0
2011	732	163 (22.3)	45	1	1	0	6	0	0
2012	57	37 (64.9)	0	0	2	0	0	0	0
2013	25	7 (28.0)	0	0	0	0	1	0	0
Rest of Chad									
2010	3,040	65 (2.1)	28	1	0	0	2	0	0
2011	5,808	353 (6.1)	110	1	1	0	0	1	0
2012	3,809	329 (8.6)	59	4	0	0	4	0	0
2013	247	99 (40.1)	1	2	0	0	12	4	4‡

*Diagnoses were based on cerebrospinal fluid (CSF) specimens received at the national reference laboratory in N'Djamena, Chad. Vaccination with serogroup A meningococcal polysaccharide/tetanus toxoid conjugate vaccine was implemented in the N'Djamena regions in 2011 and in all other regions of Chad in 2012. No reference laboratory data are available for 2009. *N.*, *Neisseria*; *S.*, *Streptococcus*; *H.*, *Haemophilus*.

†Based on culture or latex agglutination.

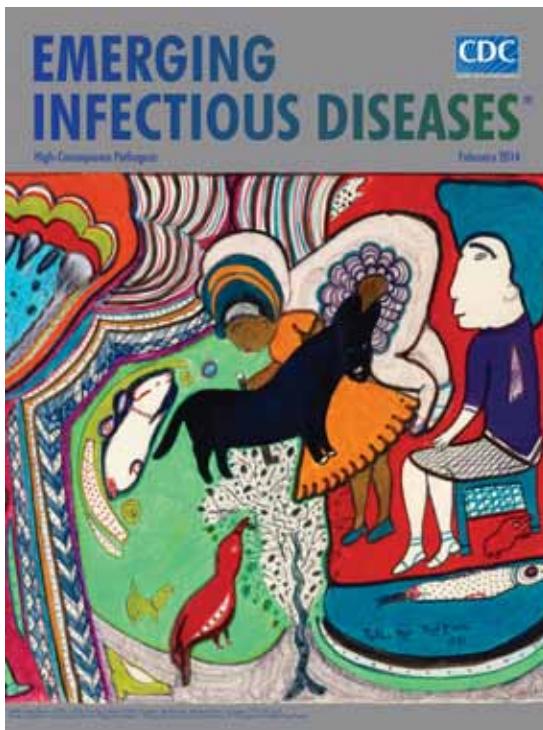
‡*Acinetobacter baumannii*, *Salmonella paratyphi* A, *Staphylococcus hominis*, *Staphylococcus aureus*.

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Increased Outbreaks Associated with Nonpasteurized Milk, United States, 2007–2012

Elisabeth A. Mungai, Casey Barton Behravesh, and L. Hannah Gould

The number of US outbreaks caused by nonpasteurized milk increased from 30 during 2007–2009 to 51 during 2010–2012. Most outbreaks were caused by *Campylobacter* spp. (77%) and by nonpasteurized milk purchased from states in which nonpasteurized milk sale was legal (81%). Regulations to prevent distribution of nonpasteurized milk should be enforced.

Pasteurization is an effective way to improve milk safety; however, in the United States, illness related to consumption of nonpasteurized milk continues to be a public health problem. The first statewide requirements that dairy products be pasteurized were enacted in Michigan in 1948 (1). In 1987, the US Food and Drug Administration banned the interstate sale or distribution of nonpasteurized milk. However, the laws regulating intrastate sales are set by each state (2). Regulations for intrastate sales of nonpasteurized milk vary from complete bans to permitting sales from farms or retail outlets (2). Even in states in which sale of nonpasteurized milk is illegal, milk can often be obtained through other means. For example, some states allow cow-share or herd-share agreements, in which buyers pay farmers a fee for the care of a cow in exchange for a percentage of the milk produced (3,4).

Consumption of nonpasteurized milk has been associated with serious illnesses caused by several pathogens, including *Campylobacter* spp., Shiga toxin-producing *Escherichia coli*, and *Salmonella enterica* serotype Typhimurium (3,4). Despite the health risks associated with consuming nonpasteurized milk, the demand for nonpasteurized milk has increased (3,5,6). Recently, many state legislatures have considered relaxing restrictions on the sale of nonpasteurized milk (2,6). We report that the number of outbreaks associated with nonpasteurized milk increased from 2007 through 2012.

The Study

A foodborne disease outbreak is defined as the occurrence of ≥ 2 cases of a similar illness resulting from ingestion of a common food. State and local health departments voluntarily report outbreaks to the Foodborne Disease Outbreak Surveillance System of the Centers for Disease Control and Prevention through a standard web-based form (<http://www.cdc.gov/nors>). We reviewed outbreaks reported during 2007–2012 in which the food vehicle was nonpasteurized milk. Outbreaks attributed to consumption of other dairy products made with nonpasteurized milk, such as cheese, were excluded. We analyzed outbreak frequency, number of illnesses, outcomes (hospitalization, death), pathogens, and age groups of patients. Data on the legal status of nonpasteurized milk sales in each state were obtained from the National Association of State Departments of Agriculture (7–9) and an online search of state regulations. The sources from which nonpasteurized milk was obtained or purchased were categorized according to the description from the state outbreak reports, when available.

During 2007–2012, a total of 81 outbreaks associated with nonpasteurized milk were reported from 26 states. These outbreaks resulted in 979 illnesses and 73 hospitalizations. No deaths were reported. The causative agent was reported for all outbreaks. Of the 78 outbreaks with a single etiologic agent, *Campylobacter* spp. was the most common pathogen, causing 62 (81%) outbreaks, followed by Shiga toxin-producing *E. coli* (13 [17%]), *Salmonella enterica* serotype Typhimurium (2 [3%]), and *Coxiella burnetii* (1 [1%]) (Figure 1). Three outbreaks were caused by multiple pathogens (Figure 1). The number of outbreaks increased from 30 during 2007–2009 to 51 during 2010–2012. During 2007–2009, outbreaks associated with nonpasteurized milk accounted for $\approx 2\%$ of outbreaks with an implicated food; during 2010–2012, this percentage increased to 5%. The number of outbreaks of *Campylobacter* spp. infection also increased, from 22 during 2007–2009 to 40 during 2010–2012 (Figure 1).

Information about the age of patients was available for 78 outbreaks (Figure 2). For 59% of outbreaks, at least 1 patient < 5 years of age was involved (Figure 2), and 38% of illnesses caused by *Salmonella* and 28% of illnesses caused by Shiga toxin-producing *E. coli* were in children 1–4 years of age (Figure 2).

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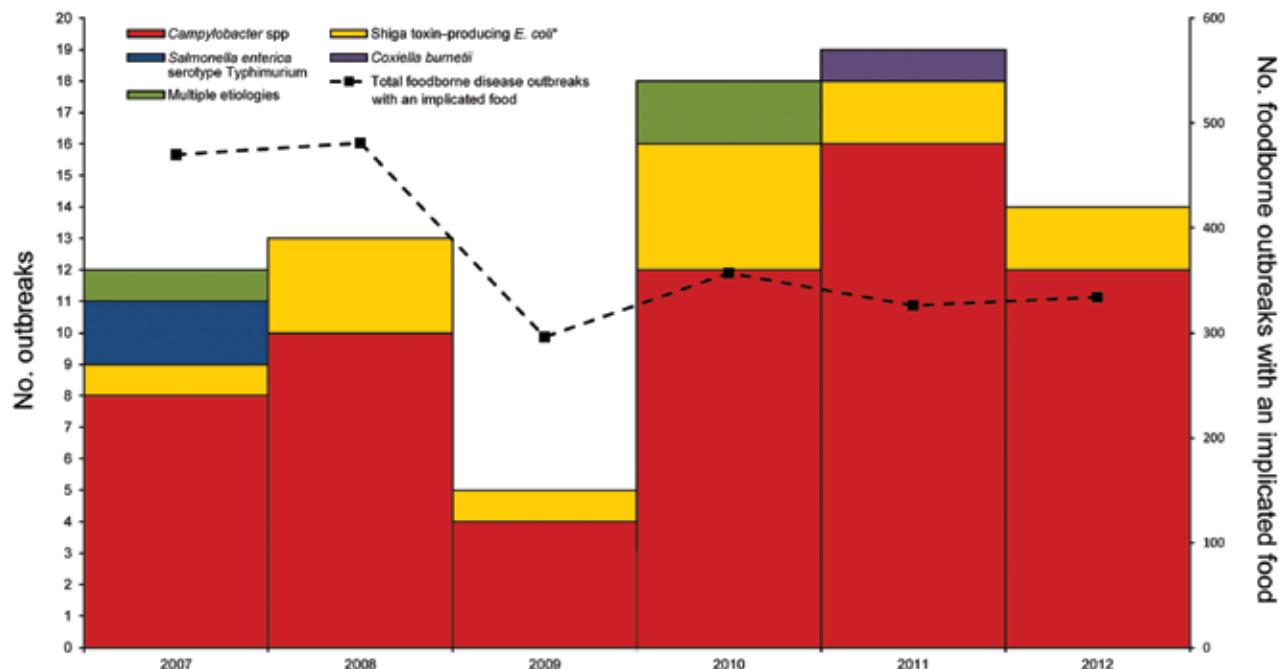


Figure 1. Outbreaks associated with nonpasteurized milk, by etiologic agent and year, United States, 2007–2012. Three outbreaks involved multiple pathogens: *Campylobacter* spp. and *Salmonella enterica* serotype Typhimurium; Shiga toxin-producing *Escherichia coli* O157:H7 and *Campylobacter*; *Campylobacter* and *Cryptosporidium*. *E. coli* serogroups: O157 (10 outbreaks), O111 (1 outbreak), O26:H11 (1 outbreak), O157:H7 and O121 (1 outbreak).

How milk was obtained was reported for 68 (84%) outbreaks. Nonpasteurized milk was obtained from dairy farms (48 [71%] outbreaks), licensed or commercial milk sellers (9 [13%]), cow- or herd-share arrangements (8 [12%]), and other sources (3 [4%]) (Table). Of the 81 outbreaks, 66 (81%) were reported from states where the sale of nonpasteurized milk was legal in some form: Pennsylvania (17 outbreaks), New York, Minnesota (6 outbreaks each), South Carolina, Washington, and Utah (5 outbreaks each) (Table). A total of 15 (19%) outbreaks were reported in 8 states in which sales were prohibited (Table). Among these outbreaks, the sources of nonpasteurized milk were

reported as a dairy farm (6 outbreaks), cow or herd share (4 outbreaks), and unknown (5 outbreaks) (Table).

Conclusions

Within this 6-year period, the number of outbreaks associated with nonpasteurized milk increased. The number of outbreaks caused by *Campylobacter* spp. nearly doubled. The average number of outbreaks associated with nonpasteurized milk was 4-fold higher during this 6-year period (average 13.5 outbreaks/year) than that reported in a review of outbreaks during 1993–2006 (3.3 outbreaks/year) (4). This increase was concurrent with a decline in the number

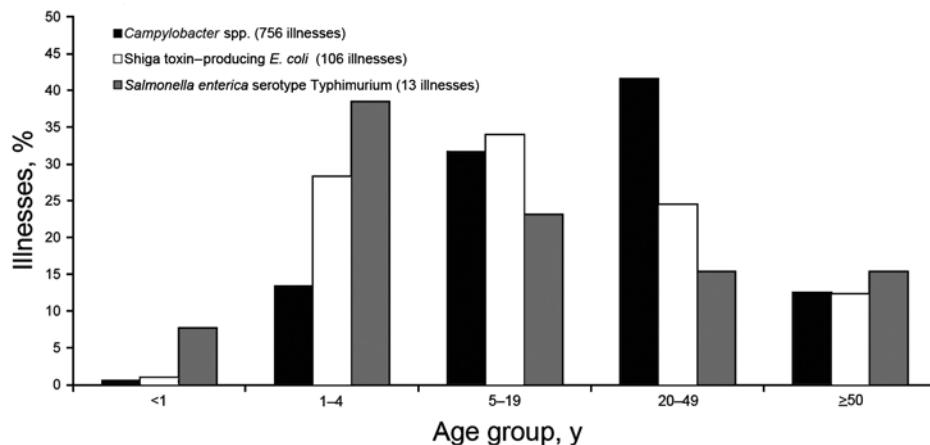


Figure 2. Percentage of patients affected by outbreaks associated with nonpasteurized milk, by age and etiologic agent, United States, 2007–2012.

Table. Source of milk in outbreaks associated with nonpasteurized milk, by legal status of state sales, United States, 2007–2012*

Legal status of raw milk sales (no. states)	State (no. outbreaks)	Source where milk was purchased or obtained†					Not reported	Total
		Dairy farm	Licensed or commercial milk seller	Cow or herd share	Other‡			
Allowed on farms and at retail stores separate from farms (legal, 12 states)	Pennsylvania (17), Washington (5), Utah (5), South Carolina (5), California (3), Idaho (1), Arizona (1), Connecticut (1)	21	7	1	3	6	38	
Restricted to farms (legal, 14 states)	Minnesota (6), New York (6), Wisconsin (3), Kansas (2), Massachusetts (1), Nebraska (1), Missouri (2)	16	2	1	0	2	21	
Allowed on farm and at retail stores if standards met (legal, 1 state)	Vermont (4)	4	0	0	0	0	4	
Only at farmer's markets (legal, 1 state)	0	0	0	0	0	0	0	
Prohibited but allows cow or herd share (1 state)	Colorado (3)	1	0	2	0	0	3	
Prohibited, including cow or herd share (illegal, 20 states)	Ohio (4), Michigan (4), North Dakota (2), Iowa (1), Indiana (1), Georgia (1), Alaska (1), Tennessee (1)	6	0	4	0	5	15	
On-farm sales allowed only from farms with 2 producing cows, 9 producing sheep, and/or 9 producing goats (legal, 1 state)	0	0	0	0	0	0	0	
Total		48	9	8	3	13	81	

*Data for this analysis were downloaded on June 6, 2013.

†Cow milk in 77 outbreaks, goat milk in 4 outbreaks.

‡Bed and breakfast lodging (1 outbreak), flea market (1 outbreak), raw milk buying club (1 outbreak).

of states in which the sale of nonpasteurized milk was illegal, from 28 in 2004 to 20 in 2011 (7–9) and with an increase in the number of states allowing cow-share programs (from 5 in 2004 to 10 in 2008) (8,9). The decision to legalize the sale of nonpasteurized milk or allow limited access through cow-share programs may facilitate consumer access to nonpasteurized milk (5). The higher number of outbreaks in states in which the sale of nonpasteurized milk is legal has been reported elsewhere (4).

The legal status of nonpasteurized milk sales in 1 state can also lead to outbreaks in neighboring states. In a 2011 outbreak of *Campylobacter* spp. infections associated with nonpasteurized milk in North Carolina, where sales of this product were prohibited, milk was purchased from a buying club in South Carolina, where sales were legal. Another outbreak of *Campylobacter* spp. infection in 2012 implicated nonpasteurized milk from a farm in Pennsylvania, where sales are legal; cases from this outbreak were reported from Maryland, West Virginia, and New Jersey, all of which prohibit sale of raw milk (10). All patients residing outside Pennsylvania had traveled to Pennsylvania to purchase the milk (10).

Outbreaks associated with nonpasteurized milk continue to pose a public health challenge. Legalization of the sale of nonpasteurized milk in additional states would probably lead to more outbreaks and illnesses. This possibility is especially concerning for vulnerable populations, who are most susceptible to the pathogens commonly found

in nonpasteurized milk (e.g., children, senior citizens, and persons with immune-compromising conditions). Public health officials should continue to educate legislators and consumers about the dangers associated with consuming nonpasteurized milk; additional information can be obtained at <http://www.cdc.gov/foodsafety/rawmilk/raw-milk-index.html>. In addition, federal and state regulators should enforce existing regulations to prevent distribution of nonpasteurized milk.

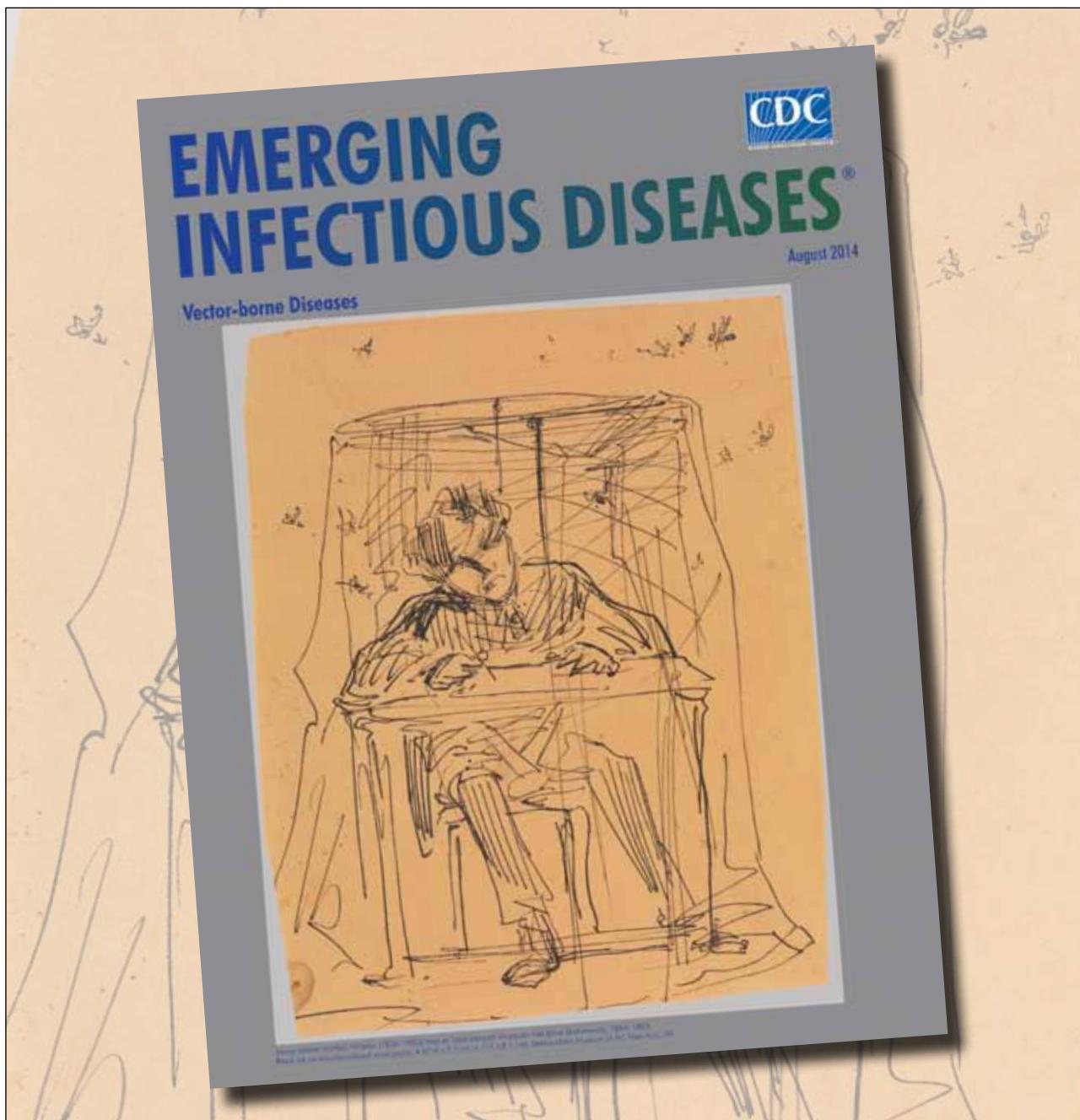
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Genetic Diversity of Enterovirus A71, India

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and Jagdish M. Deshpande

We have identified circulation of 3 genogroups of enterovirus (EV) A71 in India. A new genogroup (proposed designation G) was discovered during this study. We isolated genogroups D and G in wide geographic areas but detected subgenogroup C1 only in 1 focus in western India. A systematic nationwide search for EV-A71 is warranted.

Enterovirus A71 (EV-A71; *enterovirus species A*, genus *Enterovirus*, family *Picornaviridae*) was first isolated in 1969 from the cerebrospinal fluid of a patient with encephalitis in California, USA. EV-A71 is known to cause encephalitis; meningitis; hand, foot and mouth disease (HFMD); and acute flaccid paralysis (AFP) (1,2). EV-A71 epidemic activity has increased substantially throughout the World Health Organization South-East Asia and Western Pacific Regions since the 1997 outbreak of HFMD with severe neurologic complications and high case-fatality rates reported in Sarawak and peninsular Malaysia (3). In many countries, EV-A71 circulated for several years before large-scale outbreaks were reported (4). In China, several hundred thousand cases of HFMD have been reported in recent years (5). In India, the epidemiology of EV-A71 has remained largely unexplored. A small outbreak of HFMD in Kerala in 2003 and 36 (42%) of 87 encephalitis cases reported in western Uttar Pradesh during July 2004–November 2006 were attributed to EV-A71 infections only on the basis of serologic evidence (6,7). Isolation of EV-A71 from a patient with AFP in India was reported for the first time in 2001 (8). Recently, 2 research groups have reported frequent isolation of EV-A71 from persons with AFP in Uttar Pradesh, Karnataka, and Kerala (9,10).

EV-A71 strains isolated worldwide are classified into 4 genogroups: A–D (11). Genogroups B and C have been differentiated into subgenogroups B0–B5 and C1–C5 (11). Bessaud et al. proposed 2 new genogroups in sub-Saharan Africa (genogroup E) and Madagascar (genogroup F) (12). Other new subgenogroups proposed recently include C4a, C4b, C6, C7, and B6 (9,13). We detected 14 EV-A71 among nonpolio enterovirus (NPEV) isolates from persons with AFP, HFMD, and encephalitis reported in Mumbai and surrounding areas during 2008–2012. The objective of

this study was to evaluate the phylogenetic relationship of the Indian EV-A71 strains in the global context.

The Study

We studied 561NPEV isolates obtained from 2,530 AFP patients, 89 from 383 HFMD patients, and 1 from 23 encephalitis patients in Mumbai and surrounding areas during 2008–2012. Partial sequencing viral protein (VP1) was used to identify the NPEV (sero) types as described previously (14). Fourteen EV-A71 isolates were thus identified: 10 from AFP patients, 2 from HFMD patients, and 1 each from patients with encephalitis and febrile illness. Five EV-A71 isolates identified at various times since 2002 from northern Indian states were also included in this study (J. Deshpande, unpub. data). The VP1 region of EV-A71–positive isolates was sequenced as described previously (15). Sequencing was done by using Big Dye Terminator v3.1 Cycle Sequencing kit in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Sequences were resolved on ABI 3130xl Genetic Analyzer (Applied Biosystems) and edited by using Sequencher v4.10.1 (Gene Codes, Ann Arbor, MI, USA). Complete VP1 sequences were aligned by using ClustalW (<http://www.ebi.ac.uk>). Phylogenetic analysis was conducted in MEGA5 by using maximum-likelihood and neighbor-joining methods (<http://www.megasoftware.net>). Genetic distances were calculated by Kimura 2-parameter method. A genotype was defined by $\geq 85\%$ nt sequence similarity in the complete VP1 region (12). Sequences were deposited in GenBank under accession nos. KF906416–KF906434.

Median age of patients from whom the EV-A71 strains were isolated was 28 months; 10 (56%) were girls (Table 1). Three EV-A71–positive patients reported in Mumbai had lived in Uttar Pradesh. EV-A71–positive HFMD cases were non-neurologic, and the encephalitis patient recovered completely without neurologic deficit. Medical examination results 60 days after onset were not available for the AFP patients.

Nine isolates clustered with the strain R-13223-IND-01 (GenBank accession no. AY179600) (Figure 1), which was the only representative of EV-A71 genogroup D. A mean genetic distance of 11% and presence of multiple clusters within this genogroup indicated continuous evolution of genogroup D viruses over several years. Eight isolates (5 from AFP patients, 2 from HFMD patients, and 1 from an encephalitis patient) clustered with subgenogroup C1. Sequence similarity of $>99\%$ of 6 of the 8 isolates indicated a focal outbreak caused by C1 subgenogroup. Clustering of the subgenogroup C1

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Table 1. Characteristics of enterovirus A71–positive patients, India*

Patient ID	Age, mo/sex	Clinical disease	Onset date	Sample type tested	Onset to sample, d	Patient location, city, state
V08-179	31/M	AFP	2008 Jan 14	Feces	32	Mumbai, MH
BMC08-C20	12/M	Fever	2008 Aug 2	Feces	2	Mumbai, MH
V08-5327	37/F	AFP	2008 Nov 7	Feces	2	Mumbai, MH†
V10-4243	66/F	AFP	2010 Aug 3	Feces	38	Kanpur, UP‡
V11-913	30/F	AFP	2011 Feb 26	Feces	6	Mumbai, MH
V11-2209	40/F	AFP	2011 Apr 25	Feces	10	Jaunpur, UP‡
V11-6064	26/F	AFP	2011 Aug 2	Feces	20	Mumbai, MH
V11-4998	73/F	AFP	2011 Aug 9	Feces	12	Mumbai, MH
V11-12176	50/F	AFP	2011 Oct 13	Feces	5	Mumbai, MH
V11-12755	12/F	AFP	2011 Oct 29	Feces	2	Thane, MH
V11-H31	9/M	HFMD	2011 Nov 7	Throat swab	2	Thane, MH
V12-104	34/F	AFP	2012 Mar 8	Feces	7	Mumbai, MH
V12-CN06	42/M	Encephalitis	2012 May 28	Cerebrospinal fluid	1	Thane, MH
V12-H05	12/M	HFMD	2012 Jul 21	Throat swab	2	Thane, MH
R-17928	9/M	AFP	2002 Jun 30	Feces	9	Jotiba Phule Nagar, UP
R-19153	10/M	AFP	2002 Aug 14	Feces	9	Rampur, UP
N03-522-2	NA	Healthy child	2003 Mar 26§	Feces	0	Bulandshahr, UP
V12-1719-2	14/F	AFP	2012 Jul 7	Feces	5	Delhi, DL
R-80135	12/M	AFP	2012 Sep 11	Feces	10	Darbhanga, BI

*AFP, acute flaccid paralysis; BI, Bihar; DL, Delhi; HFMD, hand, foot and mouth disease; MH, Maharashtra; UP, Uttar Pradesh.

†Resident of Mumbai with a travel history to Azamgarh, UP.

‡Cases reported from hospitals in Mumbai.

§Fecal sample collection date for healthy child.

isolates with those from Germany, the Netherlands, and Azerbaijan suggested an epidemiologic link with the EV-A71 circulating in the European Region. V08–5327 and V11–2209 had sequence similarity of 89.5% between them and >18% divergence from all other known EV-A71 genogroups. We propose a new genogroup G designation for EV-A71 isolates V08–5327 and V11–2209. Table 2 shows estimates of evolutionary divergence of genogroup G in global context.

To study the genetic diversity among EV-A71 strains isolated in India, we extracted partial VP1 nucleotide sequences of various lengths from GenBank. We detected 50 EV-A71 strains among NPEV isolates from AFP patients in Uttar Pradesh, Kerala, and Karnataka during 2007–2009 (9). Four EV-A71 isolates were reported from AFP patients from Uttar Pradesh studied during 2009–2010 (10). We used 21 sequences of isolates from Rao (9) and 1 of Laxmivandana (10) for determining the genetic relationship of EV-A71 isolated in India. Neighbor-joining tree using partial VP1 (707 nt) sequences (Figure

2) showed that 11 EV-A71 strains assigned to genogroup F by Rao et al. actually clustered within isolates of genogroup D, and the remaining 10 EV-A71 strains clustered within the new genogroup G. None of the EV-A71 isolates of Rao et al. (9) clustered in the subgenogroup C1 (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-0743-Techapp1.pdf>).

Bessaud et al. very recently reported (12) that 20 partial sequences reported by Rao et al. did not cluster into any known subgenogroup, which suggests more genogroups in India. Our current study confirms that some of them cluster within genogroup G. The remaining sequences from the study of Rao et al. need further analysis on the basis of longer nucleotide sequences.

Conclusions

Our study reports the discovery of a new genogroup G of EV-A71 in India. Genogroups D and G may be endemic in India because they were isolated from AFP patients in wide geographic areas; however, these genogroups have not been implicated in any specific outbreaks. Moreover, the 2 genogroups appear to be indigenous to India because they have not been detected in any other country. EV-A71 strains of subgenogroup C1 were isolated only in 2011–2012, indicating very recent circulation. Subgenogroup C1 was associated with time- and space-clustered cases of AFP, HFMD, and encephalitis. Multiple genogroups and high sequence divergence within genogroups D and G showed that EV-A71 has been spreading across the country. Therefore, systematic efforts should be made to understand the impact of the virus on public health in India.

Table 2. Estimates of evolutionary divergence over sequence pairs between genogroups A–G of enterovirus A71, India*

Genogroup	Genogroup						
	A	B	C	D	E	F	G
A	X						
B	0.211	X					
C	0.195	0.205	X				
D	0.211	0.208	0.202	X			
E	0.232	0.216	0.196	0.187	X		
F	0.197	0.199	0.209	0.207	0.203	X	
G	0.224	0.206	0.187	0.172	0.193	0.197	X

*X indicates value not calculated because the diagonal values would be homologous comparisons (divergence 0%). Boldface indicates sequence divergence of genogroup G is >16% from all other genogroups.

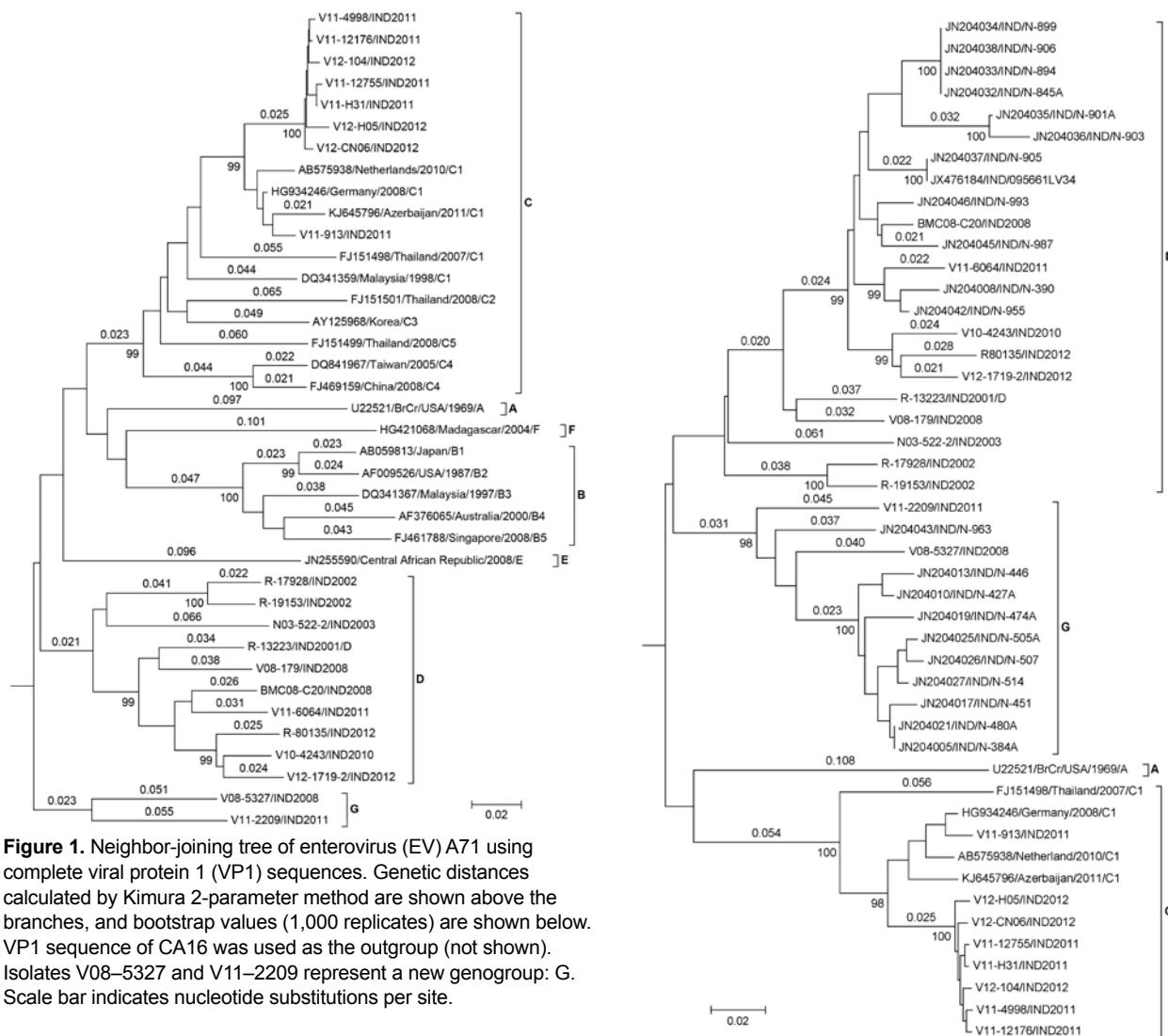


Figure 1. Neighbor-joining tree of enterovirus (EV) A71 using complete viral protein 1 (VP1) sequences. Genetic distances calculated by Kimura 2-parameter method are shown above the branches, and bootstrap values (1,000 replicates) are shown below. VP1 sequence of CA16 was used as the outgroup (not shown). Isolates V08–5327 and V11–2209 represent a new genogroup: G. Scale bar indicates nucleotide substitutions per site.

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Figure 2. Neighbor-joining tree of enterovirus (EV) A71 strains isolated in India. Partial viral protein 1 (VP1) sequences (707 nt) were used for the analysis. Genetic distances calculated by Kimura 2-parameter method are shown above the branches, and bootstrap values (1,000 replicates) are shown below. India isolates clustered into 3 genogroups D and G and subgenogroup C1. VP1 sequence of CA16 was used as the outgroup (not shown). Scale bar indicates nucleotide substitutions per site.

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Use of *Treponema pallidum* PCR in Testing of Ulcers for Diagnosis of Primary Syphilis¹

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Treponema pallidum PCR (*Tp*-PCR) has been noted as a valid method for diagnosing syphilis. We compared *Tp*-PCR to a combination of darkfield microscopy (DFM), the reference method, and serologic testing in a cohort of 273 patients from France and Switzerland and found the diagnostic accuracy of *Tp*-PCR was higher than that for DFM.

Incidence of syphilis, caused by *Treponema pallidum*, has increased steadily worldwide since the early 2000s, especially in at-risk populations (1). The US Centers for Disease Control and Prevention (CDC) recently updated the definitions for confirmed cases of primary and secondary syphilis and now considers *Treponema pallidum* PCR (*Tp*-PCR) to be a valid diagnostic method along with darkfield microscopy (DFM) (2), which is still considered the reference test (although it remains imperfect) (3). In diagnosis of sexually transmitted ulcerative disease, a positive DFM result confirms syphilis because other *T. pallidum* subspecies are not sexually transmitted and have a different geographic distribution. However, the meaning of a negative DFM result is more uncertain. Samples from up to 20% of case-patients with syphilis may show negative DFM results when the test is performed by technicians who are not fully trained or when it is performed in suboptimal conditions (3). *Tp*-PCR is clinically useful for testing of ulcers or skin lesions in areas where syphilis prevalence is high (4), but uncertainties remain because of the variability in the reference tests used in the different diagnostic studies. Moreover, the risk for misclassification by DFM diminishes the apparent value of *Tp*-PCR when DFM is the reference test because samples from syphilis patients that

yield a negative DFM result, but a positive *Tp*-PCR result, are currently considered false-positive.

We conducted a multicenter study in France and Switzerland to evaluate the accuracy of *Tp*-PCR compared with DFM and serologic testing. To resolve the difficulty of assessing a new diagnostic test against an imperfect standard, in addition to the standard DFM diagnostics, we used an enhanced definition for the diagnosis of syphilis that combines clinical information with DFM, serologic testing, or both, to enable a fair assessment to be made of the diagnostic performance of *Tp*-PCR.

The Study

We conducted a multicenter, prospective, observational study during September 2011–September 2013 in 5 centers in Switzerland and France (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-0790-Techapp.pdf>). All patients who had a genital, anal, or oral ulcer suggestive of syphilis after having at-risk sexual intercourse were invited to participate in the study. We used 3 definitions that would indicate a diagnosis of syphilis: 1) positive DFM results (5); 2) a combination of nontreponemal and/or treponemal tests as recommended by CDC (2) (if possible, samples that had negative results on a first nontreponemal assay underwent a second test to identify seroconversion [6]); and 3) an enhanced definition combining clinical information suggestive of syphilis and results from DFM and serologic testing. The diagnosis of syphilis was established by positive DFM results or negative DFM results combined with positive serologic tests as defined by the second definition, plus a clinical outcome and a drop in nontreponemal titers in response to treatment.

Clinicians collected ulcer specimens in a standardized manner. All samples were then sent to the bacteriology laboratory at the University of Geneva Hospitals, where all *Tp*-PCR testing was performed by using a previously published protocol (7) and interpreted without knowledge of the patient's clinical or serologic status.

We recruited 273 patients from the 5 centers: 140 from Paris, France; 59 from Lyon, France; 40 from Geneva, Switzerland; 17 from Lausanne, Switzerland; and 17 from Zurich, Switzerland. Patients had a mean age of 39.0 years (SD 12.2); most (252, 92.3%) were men. Mean delay from ulcer appearance to date of first medical visit was 20.4 days

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Table 1. Summary of the various indices of performance of *Tp*-PCR compared with DFM, serologic testing, or an enhanced definition for diagnosis of primary syphilis*

Reference testing	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood ratio (95% CI)		κ coefficient (95% CI)	Post-test probability (95% CI)	
			Positive	Negative		If <i>Tp</i> -PCR is positive (PPV)	If <i>Tp</i> -PCR is negative (1 – NPV)
DFM, n = 170	93.8% (79.2%–99.2%)	90.6% (84.4%–94.9%)	9.95 (5.89–16.82)	0.07 (0.02–0.26)	0.74 (0.62–0.87)	69.8% (53.9%–82.8%)	1.6% (0.2%–5.6%)
Serologic, n = 239	78.5% (68.4%–86.5%)	93.4% (88.2%–96.8%)	11.84 (6.44–21.77)	0.23 (0.16–0.35)	0.73 (0.64–0.82)	87.3% (78.0%–93.8%)	11.9% (7.3%–17.9%)
Enhanced definition, n = 170	87.5% (74.8%–95.3%)	99.2% (95.5%–100.0%)	106.75 (15.11–753.95)	0.13 (0.06–0.27)	0.90 (0.82–0.97)	97.7% (87.7%–99.9%)	4.7% (1.8%–10.0%)

**Tp*-PCR, *Treponema pallidum* PCR; DFM, darkfield microscopy; PPV, positive predictive value; NPV, negative predictive value.

(SD 33.9; n = 132). Most patients were men who have sex with men (n = 185 [71.4%]). Ulcer localization was genital (n = 148, 54.2%), anorectal (n = 98, 35.9%), or oral (n = 27, 9.9%). HIV status was known for 226 patients (82.8%); 53 were HIV positive, and 36 were receiving antiretroviral drug therapy. Nine patients received an initial HIV diagnosis at the time of the diagnostic work-up for syphilis.

DFM results were assessed for 170 patients (62.3%); 32 had positive results (18.8%). Results for 43 *Tp*-PCR specimens were positive; 13 of these were from patients who had negative DFM results. The proportion of negative DFM/positive *Tp*-PCR results was significantly higher for the 2 centers where DFM was performed only occasionally (6/15 [40.0%]) than for centers who performed DFM more often (7/155 [4.5%]; $p < 0.001$). The diagnostic performance of *Tp*-PCR against DFM was high (Table 1), and agreement between the 2 tests was substantial.

Specimens from 255 patients underwent serologic testing; 88 patients had positive results, and 16 patients had undetermined results. Results for *Tp*-PCR were less sensitive and had a lower negative predictive value when serologic tests results were used as reference than when DFM results were used as reference (Table 1). Under the enhanced definition, however, 16 patients who had negative DFM results were considered to have syphilis, and *Tp*-PCR provided higher specificity and positive predictive value when compared with this definition than when compared to either DFM or serologic test results alone (Table 1). When DFM was assessed against *Tp*-PCR and the enhanced definition (Table 2), DFM sensitivities were consistently lower. Additional results are shown in the online Technical Appendix.

Conclusions

Our results demonstrate that *Tp*-PCR has a high degree of accuracy for the definitive diagnosis of primary syphilis from lesion exudate or tissue. As expected, the clinical value of this test appeared sensitive to the choice of reference test but was hampered by misclassification errors from DFM. By definition, any discrepancy between *Tp*-PCR and DFM results has been considered primarily an error in *Tp*-PCR. However, this assumption may not always be accurate.

The reliability of DFM in our study was strongly associated with routine performance. We classified cases with negative DFM results, positive serologic results, and a clinical picture evocative of syphilis as false negatives of the DFM. When we used this definition as a reference, the diagnostic performance of *Tp*-PCR appeared higher, indicating that *Tp*-PCR has a high clinical usefulness either for confirming or for ruling out a suspicion of syphilis.

The strengths of our study are its prospective and multicenter design and the performance of *Tp*-PCR in a unique laboratory. The study sample was also representative of patients who may benefit from *Tp*-PCR in the future. The main limitation was the lack of a standard protocol for serologic testing, which could have affected the validity of some analyses. However, we attempted to minimize inter-center variability by using a blind assessment of all serologic assays by 2 experts.

Our results concur with those of Grange et al., who reported that *Tp*-PCR provides better sensitivity/specificity than DFM when compared with clinical suspicion of syphilis (8). Similarly, Heymans et al. estimated 87.0% sensitivity and 93.1% specificity of *Tp*-PCR compared with DFM (9).

Table 2. Summary of the various indices of performance of DFM compared with *Tp*-PCR or an enhanced definition for diagnosis of primary syphilis*

Reference testing, n = 170	Sensitivity (95% CI)	Specificity (95% CI)	Likelihood ratios (95% CI)		κ coefficient (95% CI)	Post-test probability (95% CI)	
			Positive	Negative		If <i>Tp</i> -PCR is positive (PPV)	If <i>Tp</i> -PCR is negative (1 – NPV)
<i>Tp</i> -PCR	69.8% (53.9%–82.8%)	98.4% (94.4%–99.6%)	44.30 (11.05–177.68)	0.31 (0.20–0.48)	0.74 (0.62–0.87)	93.8% (79.2%–99.2%)	9.4% (5.6%–15.4%)
Enhanced definition	66.7% (51.6%–79.6%)	100.0% (96.9%–100.0%)	163.33 (10.2–2615.37)	0.33 (0.22–0.50)	0.74 (0.62–0.86)	100.0% (89.3%–100.0%)	11.6% (7.3%–18.0%)

**Tp*-PCR, *Treponema pallidum* PCR; DFM, darkfield microscopy; PPV, positive predictive value; NPV, negative predictive value.

Currently, DFM is less often used in routine testing than it has been in the past (10). A survey of infectious diseases specialists found that 56% have systematically performed a rapid plasma reagin test before starting treatment for syphilis (10). Only 18% repeated the test if results were negative (10), and just 2% applied direct syndromic management (11). These numbers demonstrate a lack of consensus in the decision-making process used by experts and suggest that applying the guidelines for diagnosis of syphilis is difficult in daily practice. Moreover, although serologic testing can provide a background value for the interpretation of future tests and the assessment of treatment response, these results are often noninformative in the early phase of the infection, when up to 30% of tests return false-negative results (12).

In summary, our results confirm that using *Tp*-PCR as the reference diagnostic test for early-phase syphilis may be reasonable (2). Several arguments weigh in favor of *Tp*-PCR. First, *Tp*-PCR was more accurate than DFM when assessed against the enhanced definition in our study. Second, high-quality readings of DFM are difficult to obtain (3), especially when the test is not routinely performed. Finally, the *Tp*-PCR test relies less on human expertise than DFM, which may make *Tp*-PCR results more reproducible and testing less costly if it is performed on a routine basis.

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CME

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Fatal Legionellosis after Water Birth, Texas, USA, 2014

Elyse Fritschel, Kay Sanyal, Heidi Threadgill,
and Diana Cervantes

In 2014, a fatal infection with *Legionella pneumophila* serogroup 1 occurred in a neonate after a water birth. The death highlighted the need for infection control education, client awareness, and standardization of cleaning procedures in Texas midwife facilities.

Legionella species are the causative agents of legionellosis, an illness ranging in clinical presentation from a mild febrile illness known as Pontiac fever to a potentially fatal pneumonic condition termed Legionnaires' disease (1). *Legionella* species are ubiquitously found in the environment, and their proliferation is supported by warm water and the presence of biofilms (2). Every year, 8,000–18,000 persons are hospitalized with Legionnaires' disease in the United States (3). In Texas, USA, 763 confirmed or probable cases of *Legionella* infection were reported during 2008–2013 according to data from the National Electronic Disease Surveillance System (<http://www.cdc.gov/nndss/script/nedss.aspx>). Of these case-patients, none were <1 month of age. Despite the scarcity of reported cases of legionellosis in infants, underdeveloped lungs and immune systems place infants at high risk for severe complications. The following case report summarizes the events surrounding the death of a neonate caused by *L. pneumophila* after water birth.

The Study

In January 2014, the Texas Department of State Health Services (TDSHS) was notified of a 6-day-old infant admitted to a local pediatric hospital with loose feces, cyanosis, and respiratory failure. The infant was placed on extracorporeal membrane oxygenation because of sepsis and was prescribed ampicillin and gentamicin. Although pathogens such as *Escherichia coli*, group B *Streptococcus*, or *Listeria* were initially tested for as the suspected cause of illness, knowledge about patient exposure to a home water birth combined with symptoms of fulminant sepsis and respiratory failure led clinicians to suspect legionellosis. *Legionella* urinary antigen and PCR testing from a tracheal aspirate confirmed *L. pneumophila* serogroup 1 on day 4 of hospitalization. After 19 days of

hospitalization, the infant died. The hospital confirmed *Legionella* infection as the cause of death.

Two weeks before the child's birth, a licensed midwifery center delivered and filled a recreational-grade, jetted, soft-sided, collapsible tub with water from a private borehole well. Upon filling the tub, commercially available water purifying spa drops were added to the water. These drops are enzyme-based and do not contain chlorine. In addition, the well water had not undergone any recent filtration or chemical treatment for disinfection before use. The water circulated in the tub at $\approx 37^{\circ}\text{C}$ until 2 days before the birth. At that time, the tub was drained, re-filled with well water, and left to circulate at 37°C until the delivery. The infant was born at term by spontaneous vaginal birth with no reported complications with assistance from a certified professional midwife. After the birth, the mother was transferred to a home bathtub which had been filled with well water at the time of delivery, and the infant was held there for a short time. The mother reported a healthy pregnancy and no travel during the past 12 months.

Environmental testing of the delivery tub and the private well water source was recommended by TDSHS and conducted by an Environmental *Legionella* Isolation Techniques Evaluation laboratory certified by the Centers for Disease Control and Prevention. By the time the legionellosis was reported for public health investigation, the delivery tub had been drained, disinfected, and placed in storage before being swabbed by the midwifery center. Culture isolation results from environmental swabs of the tub and well water samples did not yield *Legionella*.

Although no environmental associations were laboratory confirmed, several measures requiring remediation were discovered during the investigation. The midwifery center used a recreational jetted tub for the birth with internal tubing that can be difficult to disinfect and that is not approved for use as medical equipment. Water treatment inside the jetted tub included a non-Food and Drug Administration-approved additive with water circulating at 37°C for an extended time. Additionally, the midwifery center did not provide any written procedures for employees or clients to follow before and during the water birth.

Conclusions

Findings from this investigation revealed a gap in the standardization and implementation of infection control practices for midwives during home water births. After reviewing available literature applicable to healthcare

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settings and contacting statewide midwifery and licensing agencies, the TDSHS drafted recommendations for the midwifery center associated with the reported fatal *Legionella* infection; TDSHS also distributed the recommendations to the licensing board in Texas, TDSHS regulatory officials, and professional midwifery organizations throughout Texas. The document provided guidance about the proper cleaning protocol for birthing tubs based on manufacturer recommendations and Centers for Disease Control and Prevention instruction (4). Recommendation was also made that recreational tubs unable to be cleaned and disinfected according these protocols should not be used. The TDSHS strongly encouraged documentation of birthing tub maintenance, including appropriate chemicals and quantities used for disinfection, as well as monitoring of pH and temperature. Additional recommendations included use of standard written procedures for employees and clients before, during, and after the water birth. These procedural documents were suggested to outline proper timing of tub filling to reduce proliferation of microorganisms, documentation of client awareness of possible risks when deviating from written procedures, and laboratory testing procedures to be followed when birthing tubs are suspected of being contaminated with *Legionella* or other pathogens.

The practice of water immersion during labor and birth has grown in popularity throughout multiple industrialized countries since the 1980s (5–7). Although midwife education about water birth exists, course curriculum and outreach are still in development. Educational and training requirements that may affect water birth infection control awareness vary by certification type, ranging from direct entry practitioners with no previous medical experience to registered practicing nurses. In a study conducted by Meyer et al. in Georgia, USA, only 30% of sampled certified nursing midwives (CNMs) had received education in their midwifery program about water birth, although most CNMs supported water birth at their facilities (8). Additionally, most CNMs were not moderately or severely worried about any disadvantages of water birth (8).

Other sporadic case reports of neonatal legionellosis after water birth have been published worldwide during the last decade (9–11). The most recent is a case of legionellosis in the United Kingdom, associated with a pre-filled, whirlpool-style heated birthing tub similar to the one used in this case report (11). The UK environmental investigation included PCR, which yielded positive results for *L. pneumophila* of the birthing tub. Although *Legionella* culture isolation is the standard method for environmental samples, decreased isolation of the organism, especially with increased holding times, has been reported (12). Use of PCR in conjunction with culture isolation

might increase the likelihood of detecting *Legionella* in environmental samples over culture isolation alone, but the detection of bacteria made nonviable by disinfection might lead to false positives (13,14). Environmental *Legionella* Isolation Techniques Evaluation certification does not yet validate for *Legionella* detection by PCR at this time. Also, limited guidance and therefore variability in sample collection techniques might greatly affect environmental isolation of *Legionella*.

Increasingly sensitive surveillance may result in additional reported cases. Continued awareness of infection potential in high-risk infants from *L. pneumophila* and other microorganisms found in potable water systems, such as *Pseudomonas aeruginosa* and *Aspergillus* spp., might help ensure a safer birthing environment through community and midwifery education and enforcement of proper infection control practices.

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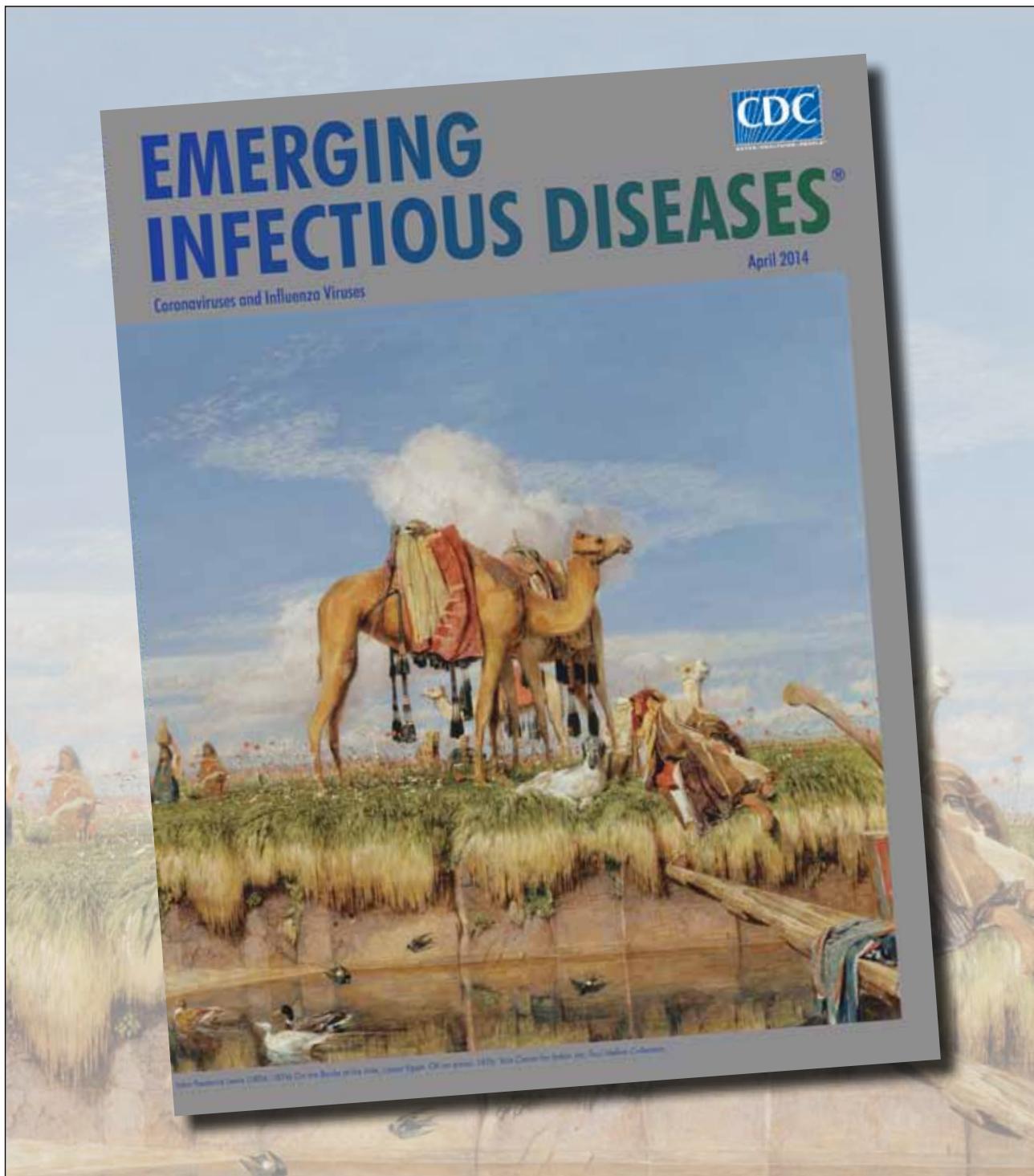
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Tularemia among Free-Ranging Mice without Infection of Exposed Humans, Switzerland, 2012

Francesco C. Origgi, Barbara König, Anna K. Lindholm, Désirée Mayor, and Paola Pilo

The animals primarily infected by *Francisella tularensis* are rapidly consumed by scavengers, hindering ecologic investigation of the bacterium. We describe a 2012 natural tularemia epizootic among house mice in Switzerland and the assessment of infection of exposed humans. The humans were not infected, but the epizootic coincided with increased reports of human cases in the area.

Although the house mouse (*Mus musculus domesticus*) is a common model for infection with *Francisella tularensis* (1), no recent and detailed data are available about natural tularemia outbreaks in this species. Tularemia mainly affects rodents and lagomorphs (2), but because these species are rapidly consumed by scavengers (3), it is challenging to conduct investigations of the biologic cycle of *F. tularensis* in the environment. Furthermore, the disease mostly occurs sporadically, although outbreaks have been reported in animals and humans (2). We describe a natural outbreak of tularemia among a population of free-ranging house mice; the epizootic occurred in Switzerland in 2012 and was associated with possible human exposure. The mouse study was approved by the Swiss Animal Experimentation Commission (Kantonales Veterinäramt Zürich; permit 51/2010).

The Study

At the edge of a forest in the Canton of Zurich, Switzerland, a population of house mice is housed in a 72-m² barn equipped with 40 nesting boxes. The population has been studied since 2002 to analyze the social structure and the population genetics of free-living house mice (4). The mice are monitored for research purposes every 2–3 days (4). Food, water, rodent bedding, and straw are provided ad libitum; mice are free to enter and exit the barn at any time. Larger animals are excluded from the barn, but other small mammals occasionally have been observed. In early June 2012, the mouse population in the barn was ≈360.

Starting in early June 2012, increased numbers of mice were found dead in the barn. During May 2012–June

2013, a total of 69 carcasses were collected and stored frozen until necropsy was performed, beginning in mid-July 2012, after the initial peak of the outbreak (Figure). Full pathologic analysis could be performed on samples from 35/69 mice, of which 15 were PCR-positive for *F. tularensis*. The primary organs were collected and processed for histologic analysis. Pathologic investigation showed the presence of macroscopic and histologic changes. Skin lesions consistent with bite and fight wounds were observed in 7 mice, 1 of which was PCR-positive for *F. tularensis*; only gram-positive cocci were detected in the associated skin lesions of this mouse by light microscopy. Splenomegaly was observed in 23 mice. In 12 of these mice, splenomegaly was secondary to tularemia, and in 8, it was associated with amyloidosis and was frequently multisystemic. In 3 mice, splenomegaly was associated with amyloidosis and *F. tularensis* infection. Red to dark red mottling of the lung was observed in several affected mice, but obvious lung hemorrhages were observed in only 2 mice. The main histologic finding was the presence of multiple foci of necrosis in spleen, liver, and lung. In addition, frequent prominent thrombi and emboli were seen in lungs in association with severe vascular inflammatory infiltration and necrosis.

Overall, lung lesions consistent with *F. tularensis* infection (necrotizing pneumonia) were seen in 67% (10/15) of the *F. tularensis*-positive mice that were examined histologically; the lesions were observed throughout the outbreak and showed various degrees of size, extension, and severity. No similar lesions were observed in any of the *F. tularensis*-negative mice. A total of 69 samples were tested for *F. tularensis* by culture and direct PCR: 49 were spleen samples, including samples from mice not selected for pathologic investigation because of severe autolysis, and 20 were abdominal swab samples, which were used when the extreme grade of autolysis prevented the unambiguous identification or collection of the spleen following dissection (5). Spleen and swab samples from 24/69 mice were positive for *F. tularensis* by PCR. Tularemia cases were observed during June–August 2012 (Figure). Eight isolates from the spleens of 8 mice were identified as *F. tularensis* subsp. *holarctica* belonging to the lineage B.FTNF002–00; these isolates shared a single multilocus variable number tandem repeat analysis profile (6,7).

During the epizootic, 11 researchers regularly entered the barn during June 1–August 31, 2012, and were

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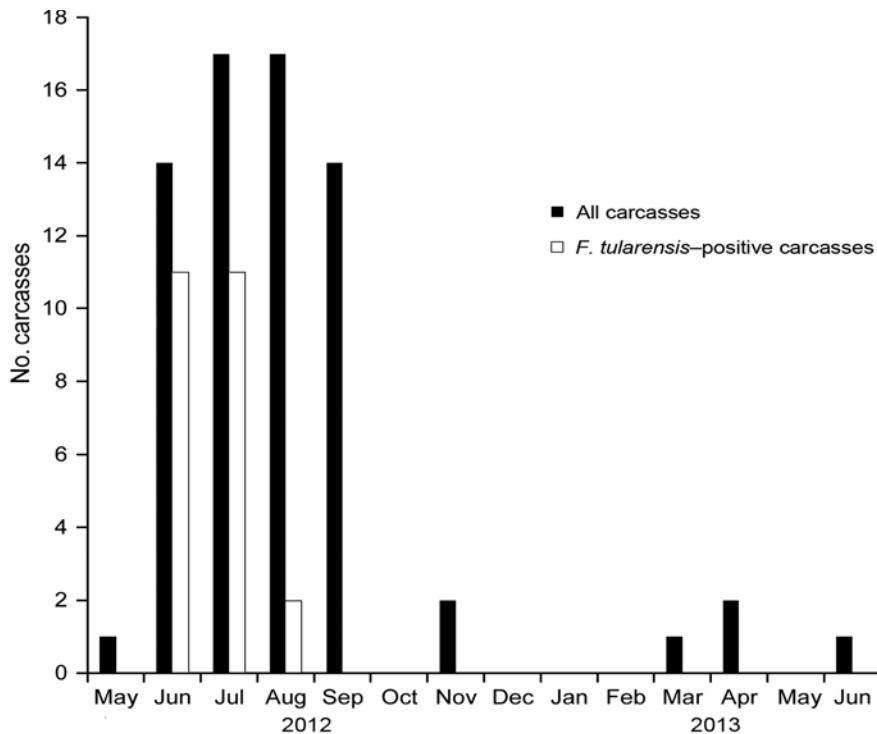


Figure. Monthly distribution of the number of carcasses of free-ranging house mice collected from a barn and the number positive for *F. tularensis*, Switzerland, May 2012–June 2013.

considered to have been exposed to *F. tularensis* (Table). On May 30 and June 29, 2012, influenza-like symptoms developed in researchers 1 and 3, respectively. *F. tularensis* antibodies persist in the blood, and serology is a standard method for diagnosing tularemia in humans. Thus, in late November or mid-December 2012 (≈ 6 months after the epizootic began), we obtained blood samples from the 11 researchers for serologic testing (VIRapid tularemia test; Vircell, Granada, Spain) (8): 10 samples were negative. The sample from researcher 3 had a positive test reaction and was further tested by microagglutination (8); dilutions of 1:40–1:640 were tested, and results were negative at 1:40. A second blood sample was obtained from researcher 3 in mid-March 2013 and was still positive by the rapid test but again showed no agglutination. Serologic cross-reaction with *Brucella* spp. was assessed and excluded. We then used the whole antigen from an outbreak isolate to perform IgM and IgG Western blots on the first and second serum samples from researcher 3: results were negative.

Conclusions

Data concerning natural outbreaks of tularemia are difficult to obtain, especially from house mice, whose carcasses rarely remain available for collection because of predators and scavengers (3). In this study, a large population of mice could be monitored under natural conditions, in the absence of antimicrobial drug treatment, during a tularemia outbreak. PCR confirmed that during the ≈ 3 -month outbreak of tularemia, 7% of the mouse population died from the disease.

This number is relatively low considering the described high sensitivity of this species to *F. tularensis* (1); however, the number of exposed mice is unknown, and not all dead mice were available for testing. The lesions observed were similar overall to those previously reported (9). However, in our investigation, lung lesions were occasionally as severe or more severe than those observed in other tissues. The lung lesions varied in size, severity, and extension but remained consistent overall, suggesting a possible single route of infection and/or systemic spread.

Cannibalism (10) might have favored the transmission of bacteria within the mouse population, but most of the carcasses with skin wounds tested negative for *F. tularensis*. Thus, transmission through cannibalism is not likely. Transmission through arthropods may be possible because

Table. Estimated time 11 researchers spent in a barn inhabited by *Francisella tularensis*-infected house mice, Switzerland, June 1–August 31, 2012

Researcher no.	Total time in barn	
	Hours	Days
1	78	22
2	49	15
3	32	4
4	29	4
5	21	4
6	18	2
7	9	1
8	9	1
9	9	1
10	9	1
11	9	1

the study population naturally harbors fleas and mites; ticks have not been observed.

Notification of *F. tularensis* outbreaks among rodents is essential, given the frequent presence of these animals in households and the consequent zoonotic potential of the pathogen (11). A unique aspect of this investigation is that we were able to evaluate humans with known exposure to infected animals. Eleven researchers entered the barn inhabited by house mice and monitored/handled the animals every 2–3 days without the use of specific personal protective equipment, except for disposable gloves; some of the mice were later found to be infected with *F. tularensis* (for more details about the monitoring/handling of animals, see [4]). The barn is a closed environment filled with bedding; mouse excrement is present on all surfaces and has the potential for aerosolization. Nevertheless, seroconversion was not detected in any of the researchers, bringing to question whether shedding of *F. tularensis* in urine and feces of mice is a key source of *F. tularensis* transmission for humans.

Thus far, reports about *F. tularensis* shedding in rodents have had inconsistent findings (12–14). However, this is a crucial point to investigate because *F. tularensis* shedding through urine and feces would not only affect outdoor environments but also household environments via rodent infestation. Moreover, in Switzerland 150% more human tularemia cases were reported in 2012 than in 2011; the increase was mostly due to cases in the same area where the barn in this study is located (15), confirming the importance of monitoring sentinel animals for tularemia to better understand the ecology of *F. tularensis*.

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Oseltamivir-Resistant Influenza A(H1N1) pdm09 Viruses, United States, 2013–14

Margaret Okomo-Adhiambo, Alicia M. Fry, Su Su, Ha T. Nguyen, Anwar Abd Elal, Elizabeth Negron, Julie Hand, Rebecca J. Garten, John Barnes, Xu Xiyan, Julie M. Villanueva, and Larisa V. Gubareva, for the 2013–14 US Influenza Antiviral Working Group¹

We report characteristics of oseltamivir-resistant influenza A(H1N1)pdm09 viruses and patients infected with these viruses in the United States. During 2013–14, fifty-nine (1.2%) of 4,968 analyzed US influenza A(H1N1)pdm09 viruses had the H275Y oseltamivir resistance-conferring neuraminidase substitution. Our results emphasize the need for local surveillance for neuraminidase inhibitor susceptibility among circulating influenza viruses.

During the 2013–14 influenza season, influenza A(H1N1)pdm09 virus was the predominant circulating virus (~80%) in the United States for the first time since the 2009 pandemic (1). We report and describe characteristics of oseltamivir-resistant influenza A(H1N1)pdm09 viruses and patients infected with these viruses in the United States.

The Study

We requested that all US state public health laboratories submit influenza-positive specimens for virologic surveillance, including antiviral susceptibility testing, as described (2). In brief, every 2 weeks each laboratory was asked to send ≤5 specimens for all virus types for virus isolation and neuraminidase (NA) inhibition assay for oseltamivir, zanamivir, and, in a subset, laninamivir and peramivir (3). All oseltamivir-resistant viruses were tested for the H275Y substitution in NA by pyrosequencing (4). Unpropagated influenza A(H1N1)pdm09 virus-positive clinical specimens were screened for the H275Y substitution by pyrosequencing (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-1006-Techapp1.pdf>). If a cluster (>2 viruses) of oseltamivir-resistant A(H1N1)pdm09 viruses was

detected, the state was asked to submit additional influenza A(H1N1)pdm09 virus specimens for testing.

We attempted to collect information by using a standard form from all patients with oseltamivir-resistant virus infection and from a sample of patients with oseltamivir-susceptible virus infection. A 2:1 (susceptible:resistant) sample was randomly selected from the list of tested specimens from the same age group in each state (<5, 5–17, 18–64, and ≥65 years). Patients with oseltamivir-resistant or -susceptible virus infections were compared by using conditional logistic regression models that controlled for age. Full NA and hemagglutinin sequence analysis was performed on all resistant viruses and a subset of susceptible viruses.

During October 1, 2013–April 30, 2014, a total of 4,968 influenza A(H1N1)pdm09 virus specimens collected from 50 US states and 2 territories were tested for antiviral susceptibility (1,811 virus isolates and 3,157 clinical specimens). A total of 59 (1.2%) influenza A(H1N1)pdm09 viruses from 20 states had the H275Y NA substitution conferring resistance to oseltamivir and peramivir (Figure 1; Table 1). None of 1,811 virus isolates was resistant to zanamivir.

Viruses with the H275Y substitution were detected in patient specimens collected during October 7, 2013–March 25, 2014; monthly prevalence ranged from 0.8% to 2.5%. Among 49 (83.0%) patients with a resistant virus infection and available information, 15 (30.6%) received oseltamivir before specimen collection (Table 2). Prior oseltamivir use was more frequent among hospitalized patients and patients with resistant virus infections than those with susceptible virus infections. Among those with prior exposure, 6 (40.0%) patients with oseltamivir-resistant and none with oseltamivir-susceptible virus infections were immunocompromised ($p = 0.03$). No differences were found between patients with oseltamivir-resistant or -susceptible virus infections.

Most resistant viruses were clustered in 5 states (California, Hawaii, Louisiana, Mississippi, and Pennsylvania) (Figure 1). Among patients with oseltamivir-resistant virus infection, only 1/4 from California, 0/4 from Hawaii, 3/11 from Louisiana, 1/3 from Mississippi, and 0/14 from Pennsylvania had exposure to oseltamivir before specimen collection. All patients from Pennsylvania except 1 attended 1 of 2 universities (among 7 participating students, none shared classes, residences, or social events). There were no epidemiologic links between other patients.

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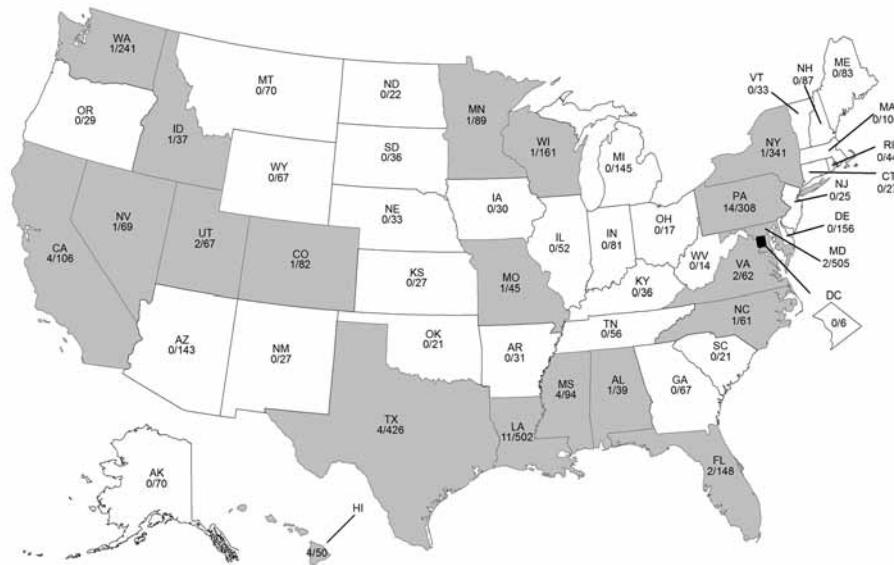


Figure 1. Geographic distribution of oseltamivir-resistant influenza A(H1N1)pdm09 viruses, United States, 2013–14. Gray indicates the presence of an oseltamivir-resistant virus. Number of oseltamivir-resistant A(H1N1)pdm09 viruses divided by total number of viruses tested is shown for each state. Oseltamivir-resistant A(H1N1) pdm09 viruses were significantly more prevalent in Louisiana (LA) ($p = 0.04$, by Fischer 2-sided exact test), Pennsylvania (PA) ($p < 0.001$), Mississippi (MS) ($p = 0.02$), Hawaii (HI) ($p = 0.02$), and California (CA) ($p = 0.03$) than in all other states combined.

Limited information was available for oseltamivir-treated patients with resistant and susceptible virus infections (online Technical Appendix Table).

Most hemagglutinin sequences from US influenza A(H1N1)pdm09 viruses collected since October 1, 2013, belonged to the 6B genetic group, and there was minimal separate clustering between susceptible and resistant viruses (online Technical Appendix Figure 1). Similar results were observed for the phylogenetic tree of the NA gene (Figure 2). NA sequences from resistant viruses in the United States with the H275Y substitution were generally scattered among other susceptible viruses from genetic group 6B viruses. Most (>99%) influenza A(H1N1)

pdm09 viruses currently in circulation have NA substitutions V241I and N369K (online Technical Appendix Figure 2). There was >1 cluster of NA sequences with the N386K substitution; each cluster contained susceptible and resistant viruses. Most (>89%) resistant viruses from the United States do not have the N386K mutation.

Conclusions

During the 2013–14 influenza season, prevalence of oseltamivir-resistant influenza A(H1N1)pdm09 viruses was low ($\approx 1\%$) in the United States, although prevalence was higher in a few states. Most patients infected with an oseltamivir-resistant influenza A(H1N1)pdm09 virus had no

Table 1. Neuraminidase inhibitor susceptibility for influenza A(H1N1)pdm09 viruses, United States, October 1, 2013–April 30, 2014*

Method of testing	Neuraminidase inhibitor			
	Oseltamivir	Zanamivir	Peramivir	Laninamivir
Neuraminidase inhibition assay†				
No. virus isolates tested‡	1,811	1,811	1,431	352
No. oseltamivir susceptible (mean $IC_{50} \pm SD$), nmol/L	1,792 (0.19 ± 0.14)	1,811 (0.18 ± 0.06)	1,412 (0.06 ± 0.02)	352 (0.23 ± 0.08)
No. oseltamivir resistant (mean $IC_{50} \pm SD$), nmol/L	19 (181.31 ± 67.63)	0	19 (17.71 ± 6.83)	0
Resistance, %	1.1	0	1.3	0
Pyrosequencing§				
No. clinical specimens tested	3,157	NA	3,157	NA
No. H275 wild-type	3,117	NA	3,117	NA
No. H275 variants	40	NA	40	NA
Resistance, %	1.3	NA	1.3	NA
Total				
No. virus tested	4,968	1,11	4,588	352
No. resistant viruses	59	0	59	0
Resistance, %	1.2	0	1.3	0

* IC_{50} , 50% inhibitory concentration; NA, not applicable.

†H275Y confirmed in virus isolate by pyrosequencing and full neuraminidase sequencing.

‡Most (99.8%) influenza A(H1N1)pdm09 virus isolates were characterized as A/California/7/2009-like, the influenza A (H1N1) component of the 2013–2014 Northern Hemisphere influenza vaccine.

§Includes pyrosequencing data from New York contract laboratory and data submitted by 19 state public health laboratories in Arizona, California, Colorado, Delaware, Florida, Georgia, Hawaii, Idaho, Maine, Maryland, Massachusetts, Michigan, Minnesota, New York, Pennsylvania, Texas, Utah, Washington, and Wisconsin.

Table 2. Characteristics of patients infected with oseltamivir-resistant and -susceptible A(H1N1)pdm09 viruses, United States, October 1, 2013–April 30, 2014*

Characteristic	Patients with oseltamivir-resistant infections, n = 49†	Patients with oseltamivir-susceptible infections, n = 93†	p value	OR (95% CI)
Median age, y (IQR)	25 (14–53)	24 (18–46)	0.86	NA
Male sex	22/49 (45)	37/92 (40)	0.59	1.21 (0.59–2.46)
White race	32/47 (68)	64/88 (73)	0.84	0.73 (0.37–1.89)
Exposure to oseltamivir before specimen collection	15/49 (31)	9/93 (10)	0.002	4.12 (1.65–10.31)
Outpatients	2/32 (6.3)	1/65 (1.5)	0.21	4.27 (0.37–48.9)
Hospitalized patients	13/17 (76)	8/27 (30)	0.003	7.72 (1.92–31.06)
Exposure to others in household receiving antiviral drugs before patient's illness	5/33 (15)	4/62 (6)	0.16	2.64 (0.63–11.07)
Any underlying medical conditions	25/49 (51)	50/93 (54)	0.8	0.85 (0.39–1.86)
Chronic pulmonary disease	5/49 (10)	7/93 (8)	0.59	1.2 (0.28–5.07)
Chronic cardiac disease	6/49 (12)	10/93 (11)	0.79	1.38 (0.39–4.83)
Diabetes mellitus	6/49 (12)	8/93 (9)	0.49	1.82 (0.53–6.26)
Immunosuppressive conditions‡	8/49 (16)	6/93 (7)	0.07	3.2 (0.1–10.3)
Other§	9/49 (18)	23/93 (25)	0.39	0.66 (0.27–1.61)
Hospitalized during influenza illness	17/49 (35)	27/92 (29)	0.51	1.51 (0.67–3.43)
Patient died	7/47 (15)	6/93 (6)	0.1	2.8 (0.86–9.14)
Others in the household were ill before patient's illness	12/32 (38)	21/65 (32)	0.61	1.2 (0.47–3.05)

*OR, odds ratio adjusted for age group; IQR, interquartile range; NA, not applicable.

†Values are no./total (%) unless otherwise indicated.

‡Long history of steroids treatment, HIV/AIDS, solid organ transplant, lupus, solid tumor malignancy, hypothyroidism, leukemia, and pituitary condition.

§Morbid obesity, chronic liver disease, neurologic disorders, chronic kidney disease, seizure, epilepsy, and depression.

prior exposure to oseltamivir. These findings are consistent with a low, and locally variable, level of circulation of resistant viruses. In our study, exposure to oseltamivir before specimen collection was more common among hospitalized patients with resistant virus infections than those with susceptible virus infections. We cannot differentiate whether these viruses emerged during treatment or were present before treatment, but many patients were immunocompromised, a condition associated with emergence of resistance during treatment (5).

Before 2007, resistance to NA inhibitors among influenza viruses circulating globally was low (<1%) (6). However, the 2007–08 influenza showed an emergence of oseltamivir-resistant seasonal influenza A(H1N1) H275Y viruses at variable prevalence (6), and by the 2008–09 season, many countries were reporting up to 100% oseltamivir resistance (7). The sharp increase in seasonal influenza A(H1N1) H275Y viruses from <1% to ≈100% was not attributed to oseltamivir use (8), but was probably caused by evolutionary advantage of H275Y variants. Studies suggest that permissive NA mutations, including R222Q, V234M, and D334N, counteracted the detrimental effect of H275Y on NA function and virus replicative properties, thus enabling virus to remain fully functional (9). The exact mechanism(s) responsible for evolutionary advantage of seasonal influenza A(H1N1) H275Y viruses over oseltamivir-susceptible viruses remain unknown.

Since emergence of influenza A(H1N1)pdm09 virus in 2009, there is concern that the H275Y substitution may become fixed in the viral genome, as it did in seasonal influenza A(H1N1) virus in 2008–09. Oseltamivir-resistance

among influenza A(H1N1)pdm09 viruses during their first 2 seasons in circulation (2009–11) remained low (<1%) (2,5). However, during June–August 2011, in Newcastle, New South Wales, Australia, a cluster of oseltamivir-resistant influenza A(H1N1)pdm09 H275Y viruses was detected among patients without prior oseltamivir exposure (10), suggesting community transmission. These H275Y viruses had permissive mutations, V241I and N369K, in addition to N386S (10), which was similar to H275Y viruses isolated in 2012 from Dutch travelers returning from Spain (11). These mutations were believed to offset the destabilizing effect of H275Y and possibly enhance virus transmissibility. The substitutions V241I, N369K, or N386S were not present in influenza A(H1N1)pdm09 virus when it emerged in 2009. However, since 2011, circulating influenza A(H1N1)pdm09 viruses have acquired these substitutions, coinciding with increasing evidence for community transmission of influenza A(H1N1)pdm09 H275Y viruses in the United States and other countries (2,12).

All influenza A(H1N1)pdm09 viruses circulating in the United States in 2013–14 had V241I and N369K substitutions, and ≈10% of resistant viruses and ≈20% of susceptible viruses had an additional substitution (N386K). All influenza A(H1N1)pdm09 H275Y viruses detected in China and Japan in 2013–14 had all 3 substitutions (13). In combination with the H275Y substitution, V241I or N369K enhances surface expression and activity of NA (14). The N386S substitution and the recently detected N386K substitution result in loss of a glycosylation site (15). Although the potential role of these changes in virus spread was suggested (10), no direct evidence is available.

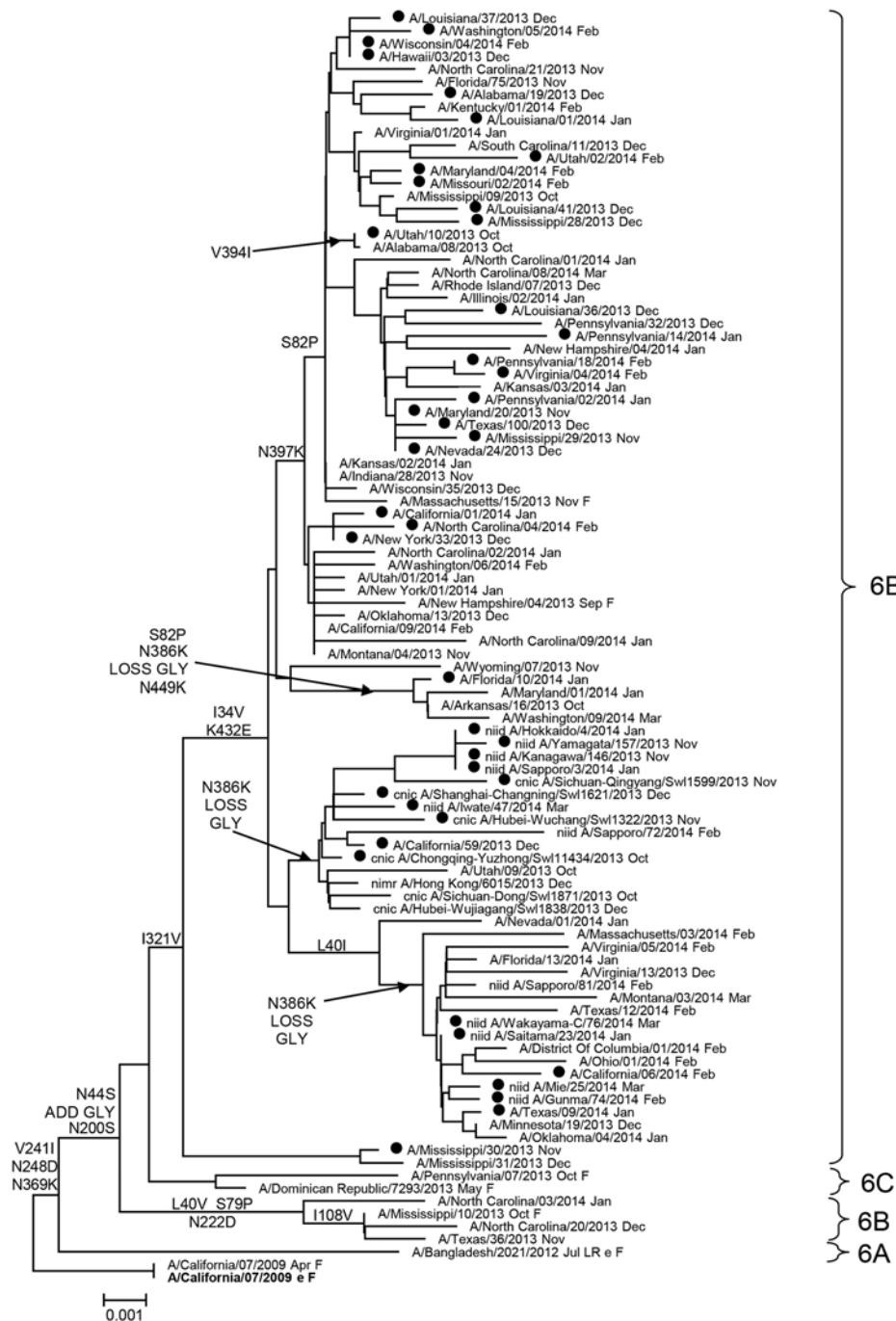


Figure 2. Evolutionary relationships among influenza A (H1N1)pdm09 virus neuraminidase genes, United States, 2013–14. Phylogenetic tree was generated by using MEGA software v5.2 (<http://www.megasoftware.net/>) and the neighbor-joining method. Evolutionary distances were computed by using the maximum composite likelihood model. Analysis included 100 representative A(H1N1)pdm09 neuraminidase gene sequences. Scale bar indicates nucleotide substitutions per site. Solid circles indicate oseltamivir-resistant H275Y markers. A/California/07/2009 (current Northern Hemisphere vaccine strain) virus was used as a reference for ancestry (root) and numbering. F, Centers for Disease Control and Prevention reference antigen; Oct, October 2013; Nov, November 2013; Dec, December 2013; Jan, January 2014; Feb, February 2014; GLY, glycosylation.

Close monitoring for the N386K/S substitution may provide information needed to delineate its role in virus spread. In addition to permissive NA mutations, other properties, such as antigenic novelty, which might provide an advantage to oseltamivir-resistant viruses and facilitate their spread, should also be monitored.

The potential for emergence and spread of oseltamivir-resistant influenza A(H1N1)pdm09 viruses, coupled with limited pharmaceutical options against influenza,

emphasizes the need for local surveillance for NA inhibitor susceptibility among circulating influenza viruses. Studies on biologic characteristics (e.g., replication in and transmissibility from ferrets) of influenza A(H1N1)pdm09 virus community isolates with H275Y and other permissive mutations are ongoing.

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Avian Influenza (H7N9) Virus Infection in Chinese Tourist in Malaysia, 2014

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Of the ≈400 cases of avian influenza (H7N9) diagnosed in China since 2003, the only travel-related cases have been in Hong Kong and Taiwan. Detection of a case in a Chinese tourist in Sabah, Malaysia, highlights the ease with which emerging viral respiratory infections can travel globally.

Human infection with avian influenza (H7N9) virus was first reported from China in 2013 (1). Since then, ≈400 cases have been diagnosed in China and some in Hong Kong and Taiwan. Most patients were older adults with severe community-acquired pneumonia; risk for admission to an intensive care unit is 83%, and risk for death is 27%–36% (2,3). We report avian influenza (H7N9) virus infection outside greater China, in a Chinese tourist visiting Sabah, Malaysia.

The Study

The patient was a 66-year-old woman from Guangzhou, Guangdong Province, China. She was not obese and had no relevant medical history. She worked on a household farm that cultivated vegetables but not animals. On January 30, 2014, she purchased from a poultry market 3 live chickens, which she slaughtered and cooked that day. According to the patient, these chickens and those at the market appeared healthy; she reported no contact with other birds. Two days later, she experienced cough, myalgia, and fever and consulted a local doctor who treated her symptomatically without performing laboratory or

radiologic investigations. The woman subsequently went on holiday and flew to Peninsular Malaysia on February 4 and to Sabah, Malaysian Borneo, on February 6. She had persistent fever, worsening productive cough, arthralgia, abdominal pain, and diarrhea. On February 7, she sought care at Tuaran District Hospital for acute respiratory distress; she was intubated and transferred to a private specialist hospital in Kota Kinabalu, Sabah, Malaysia. At admission, she was placed in a negative-pressure isolation room with a portable high-efficiency particulate air filter that removes air particles $\geq 0.3 \mu\text{m}$; staff observed airborne-transmission precautions.

Abnormal findings during initial examination were blood pressure 70/40 mm Hg, heart rate 96 beats/minute, and generalized crackles heard on lung auscultation. Hematologic, biochemical, and arterial blood gas results from samples obtained at admission (while the patient received 100% oxygen by mechanical ventilation) are summarized in the Table. Chest radiographs showed extensive dense pulmonary consolidations. The patient received dopamine, ceftriaxone, azithromycin, and oseltamivir. Culture of blood collected at admission grew methicillin-susceptible *Staphylococcus aureus*; tracheal aspirate results were negative, and cloxacillin was administered. The patient received ventilatory support with synchronized intermittent mandatory ventilation with a positive end-expiratory pressure of 10 cm H₂O. On February 13 she was given methylprednisolone, which was discontinued on February 20 and replaced with oral prednisone on February 21. Ventilatory requirements gradually decreased, and she was extubated on February 22, after blood results normalized and oseltamivir was discontinued. However, on February 23, a low-grade fever and *Pseudomonas aeruginosa* bacteremia were found, and the patient was given meropenem and piperacillin-tazobactam. On February 28, her respiratory symptoms and fever recrudesced after discontinuation of oral prednisone. On the same day, oseltamivir and prednisone were given along with inhaled zanamivir, intravenous cefepime, and ciprofloxacin; her condition gradually improved. On March 7, oseltamivir and zanamivir were discontinued; on March 13, the patient was considered well and was discharged with a tapering dose of prednisone. She returned to China on March 16. A time line of her travel and hospital course is detailed in the Figure.

According to Malaysian Ministry of Health criteria, infection with influenza (H7N9) virus is suspected in persons with severe respiratory illness and a history of travel from affected areas in China. For this patient, on February

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Table. Hematologic and biochemical values for Chinese patient with avian influenza (H7N9) virus infection at hospital admission, Malaysia, February 7, 2014

Laboratory test	Value (reference range)
Blood counts	
Hemoglobin, g/dL	12.7 (12.0–18.0)
Leukocyte, cells/mm ³	10.2×10^3 ($3.5\text{--}12 \times 10^3$)
Neutrophils, cells/mm ³	9.2×10^3 ($2.5\text{--}7.5 \times 10^3$)
Lymphocytes, cells/mm ³	276 ($1.0\text{--}4.8 \times 10^3$)
Platelets, cells/mm ³	150×10^3 ($150\text{--}400 \times 10^3$)
Serum tests	
Creatinine, mmol/L	87 (50–110)
Sodium, mmol/L	140 (135–145)
Potassium, mmol/L	3.6 (3.5–5.1)
Chloride, mmol/L	102 (96–106)
Total protein, g/L	57 (60–80)
Albumin, g/L	23 (35–50)
Globulin, g/L	34 (25–40)
Total bilirubin, μ mol/L	6.6 (2–28)
Alanine aminotransferase, IU/L	92 (7–40)
Aspartate aminotransferase, IU/L	208 (5–35)
C-reactive protein, mg/L	263.76 (<3)
Arterial blood measurements*	
pH	7.41 (7.35–7.45)
Partial pressure of oxygen, mm Hg	65 (75–100)
Partial pressure of carbon dioxide, mm Hg	4.3 (38–42)
Lactate, mmol/L	1.4 (0–2)

*Obtained while patient was receiving 100% oxygen.

7, clinicians from district and specialist hospitals sent throat swabs in viral transport media (Copan Diagnostics, Inc., Murrieta, CA, USA) to the National Influenza Centre at the Institute of Medical Research in Kuala Lumpur for virus studies. Real-time reverse transcription PCR was used to test for the following viruses, as described (4): influenza A, influenza B, influenza A(H1N1) pdm09, influenza H1 seasonal, influenza H3 seasonal (all by using Centers for Disease Control and Prevention protocols [5]), influenza H7 avian (Centers for Disease Control and Prevention, Chinese National Influenza Centre [1], and local protocols), and influenza N9 (Chinese National Influenza Centre protocol). Results were positive for influenza A and influenza H7 and N9: results remained positive on repeated swab samples collected on February 13 and 22. Sequencing of the hemagglutinin and neuraminidase genes revealed that these strains were closely related to strains from Guangzhou and Guangdong (4) but did not reveal the mutation associated with oseltamivir resistance: R294K (N9 numbering) (6). No virus was detected in samples collected on February 26 and 28.

During February 7–16, officials from the Malaysian Public Health Department and Sabah State Health Department identified 191 persons who had had contact with the patient; 6 were symptomatic, but nasopharyngeal swab specimens were negative for influenza. The symptomatic contacts included tour group members and personnel from various hotels, restaurants, airlines, tourist destinations, airports, and hospitals.

Conclusions

The rapid diagnosis of avian influenza (H7N9) virus infection outside China and Taiwan in a Chinese tourist traveling to Sabah, Malaysia, highlights the value of a high index of suspicion by medical staff, awareness and adherence to national guidelines, and good laboratory services (4). The clinical features of the patient were similar to those previously reported from China: median age of affected persons was 61 years, and >80% of patients reported exposure to live poultry and experienced pneumonia or respiratory failure (7). Virus was detected by reverse transcription PCR after the patient had received oseltamivir for 2 weeks, as has been reported (6), although the significance of prolonged detection is unclear because the patient's clinical condition improved markedly, no oseltamivir resistance mutations were found (4), and virus persistence may have been associated with steroid use. The patient's condition deteriorated after taking oseltamivir 1 week after symptom onset and ventilatory requirements increased, possibly because of secondary *S. aureus* infection, a well-known complication of influenza (8). The patient was given methylprednisolone after a week of hospitalization; although she subsequently improved, it was unclear if this was in response to the antimicrobial drugs, the corticosteroids, or the natural course of the infection. Anecdotal reports describe clinical improvement of a patient in Taiwan with influenza (H7N9) pneumonia after receipt of corticosteroids (9), but such improvement has not been supported by larger studies of influenza (H7N9) patients (3) or studies of adults in Vietnam infected with avian influenza (H5N1) virus (10,11).

Of the \approx 400 cases of human avian influenza (H7N9) infection diagnosed in China since 2003, the only travel-related cases were in Hong Kong and Taiwan, which have close geographic, economic, and cultural ties to China and extensive bidirectional travel. This case highlights the ease with which emerging viral infections can travel globally. On a map of recent air travel from China, major destinations identified (12) were Taiwan, Hong Kong, Malaysia, and Singapore; dozens of flights went to major cities in Europe and North America, which received hundreds of visitors directly from China weekly. In the first 3 quarters of 2013, an estimated 72.5 million tourists left China (13). To ensure accurate identification and appropriate management of emerging novel respiratory viral infections, clinicians in destination countries need to obtain detailed travel histories from tourists and returning travelers.

The influenza (H7N9) virus is not easily transmissible among humans, and our investigations did not find any evidence of spread to the patient's fellow travelers, medical staff, or other contacts. However, the virus has the potential to adapt to mammalian hosts over time (14,15). Clinicians and public health authorities need to be alert to the latest

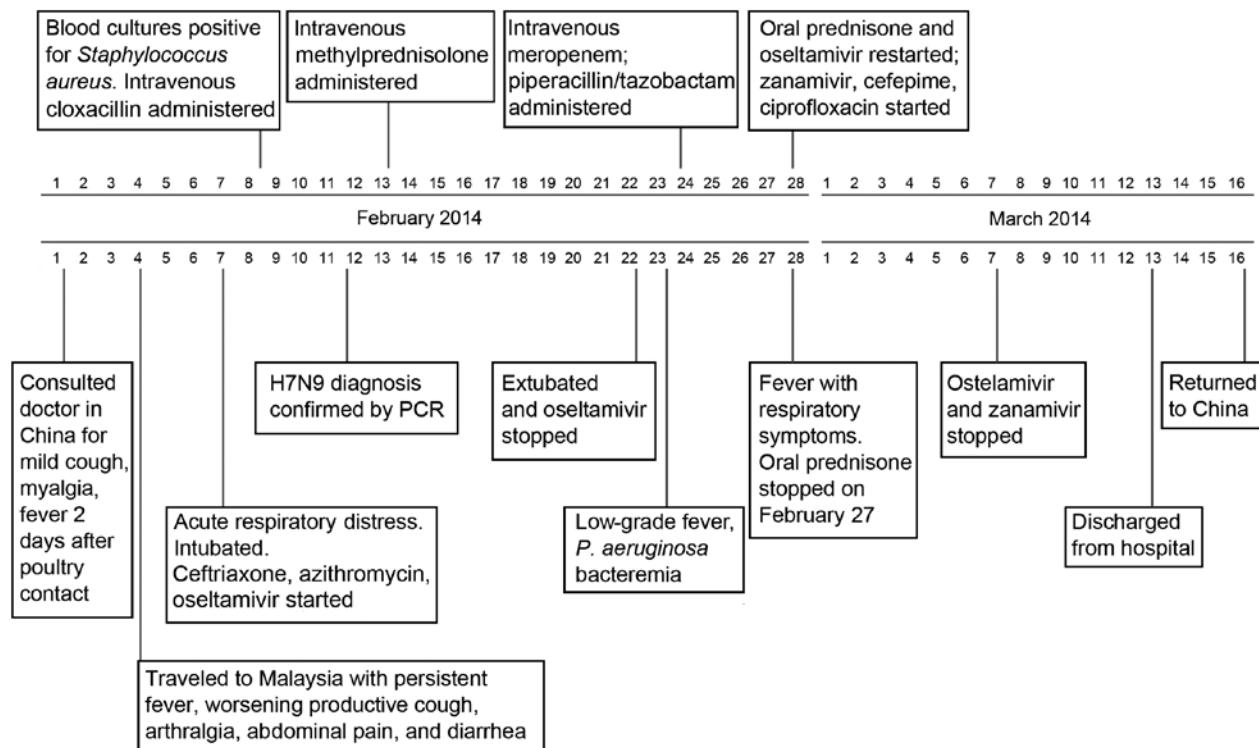


Figure. Time line of the travel dates and hospital course of avian influenza (H7N9) virus infection in Chinese tourist to Malaysia.

epidemiologic information on emerging respiratory viruses; local capacity to isolate, diagnose, and treat illness in travelers with unusual respiratory viral infections is also needed.

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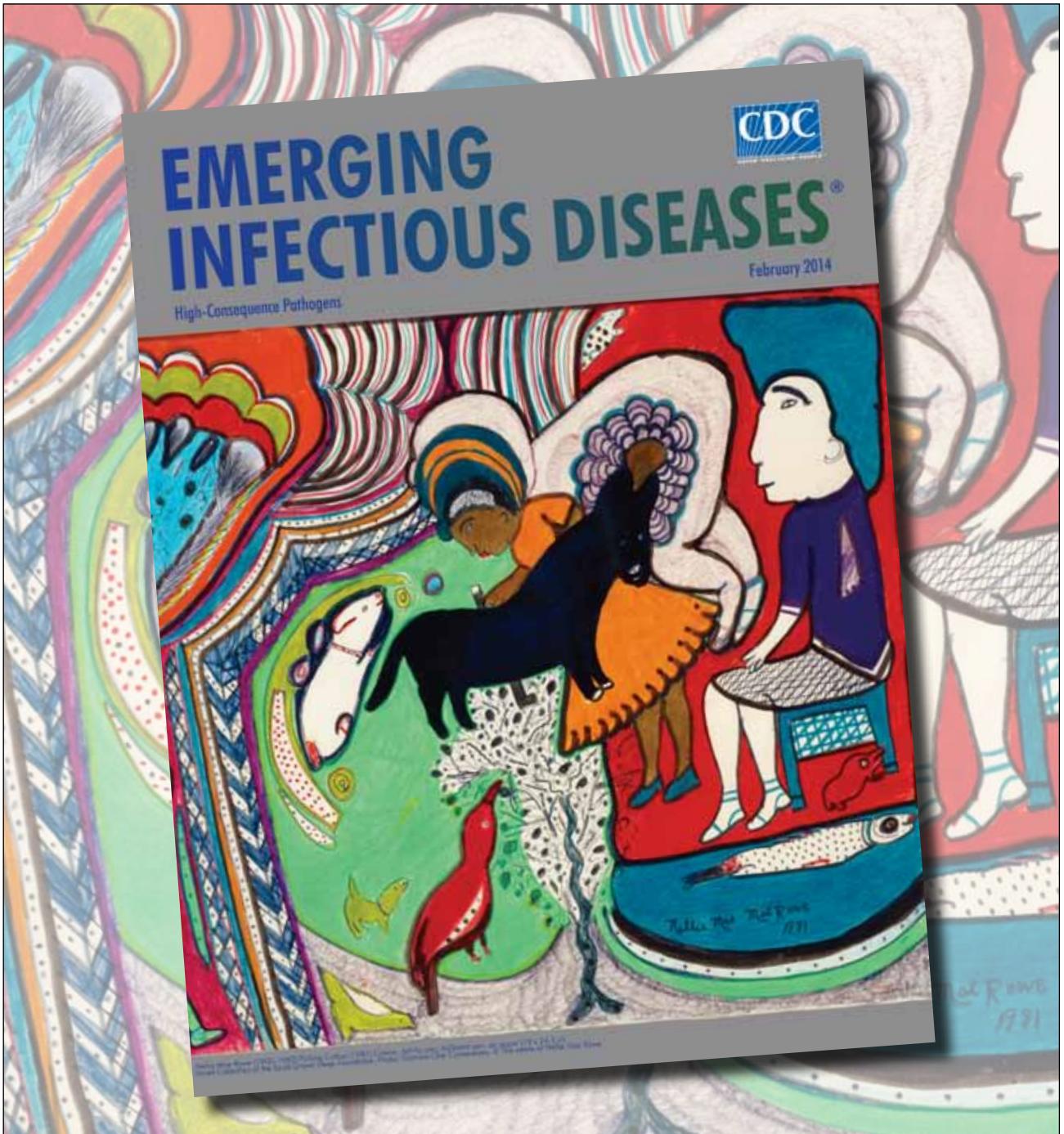
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Kyasanur Forest Disease Outbreak and Vaccination Strategy, Shimoga District, India, 2013–2014

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We investigated a Kyasanur Forest disease outbreak in Karnataka, India during December 2013–April 2014. Surveillance and retrospective study indicated low vaccine coverage, low vaccine effectiveness, and spread of disease to areas beyond those selected for vaccination and to age groups not targeted for vaccination. To control disease, vaccination strategies need to be reviewed.

In India, Kyasanur Forest disease (KFD), a tickborne viral hemorrhagic fever that occurs as seasonal outbreaks during January–June (1,2), has been endemic to 5 districts of Karnataka State. However, during 2012–2013, KFD infection was reported from other districts and states in India: Chamarajanagara District, Karnataka State; Nilgiri District, Tamil Nadu State; and Waynad District, Kerala State (3).

Vaccination with formalin-inactivated tissue-culture vaccine has been the primary strategy for controlling KFD. The strategy involves mass vaccination in areas reporting KFD activity (i.e., laboratory evidence of KFD virus [KFDV] in monkeys, humans, or ticks) and in villages within a 5-km radius of such areas (Directorate of Health and Family Welfare Services, Government of Karnataka, 2005 manual on Kyasanur Forest disease; unpub. data). Two vaccine doses are administered at least 1 month apart to persons 7–65 years of age. Vaccine-induced immunity is short-lived, so the first booster dose of vaccine is recommended within 6–9 months after primary vaccination; thereafter, annual booster doses are recommended for 5 years after the last confirmed case in the area (4).

Beginning in January 2014, increased cases of unexplained fevers were reported from Thirthahalli Taluk, a subdistrict of Shimoga District (Figure 1). On February

6, 2014, the National Institute of Virology (Pune, India) confirmed the presence of KFDV in 5/12 serum samples from patients. We investigated the outbreak to describe the epidemiologic characteristics of KFD, estimate vaccine effectiveness (VE) and coverage, and propose recommendations for control.

The Study

We established KFD surveillance in 3 large public health facilities in Thirthahalli Taluk: Thirthahalli Taluk Hospital, Kannangi Community Health Center, and Konandur Primary Health Center. Patients from neighboring Hosanagara Taluk also seek care at these facilities. A suspected case was defined as sudden onset of fever, headache, and myalgia in patients attending these facilities during the last week of December 2013 through the first week of April 2014. Medical officers at surveillance facilities collected information regarding each patient's age, sex, place of residence, and clinical and vaccination history. Serum samples from all suspected case-patients were tested for KFDV at the National Institute of Virology by using reverse transcription PCR (RT-PCR) (5). RT-PCR-negative samples were tested by ELISA for KFDV IgM (5). Samples were also tested for dengue virus and *Leptospira* spp. We analyzed the data to describe the disease by time, location, and person. We used the population of affected villages and applied the age- and sex-distributions of Karnataka State's population (2011 census) to the population of the affected villages to calculate attack rates.

To calculate vaccine coverage, we obtained 2013 KFD vaccination data from district health officials. To estimate VE, we conducted a retrospective cohort study in 4 villages within the Kannangi Community Health Center catchment area: Garaga-Kikkeri (466 residents in 106 households), where KFD vaccination was conducted in 2013, and the neighboring villages of Kannangi, Kombinakai, and Avalagere (total of 528 residents in 146 households), where vaccination was not conducted. We systematically sampled 60 households from the vaccinated village and 110 from the nonvaccinated villages and collected information from persons 8–66 years of age about the number of KFD vaccine doses received in 2013. Information about laboratory-confirmed cases in vaccinated and unvaccinated persons was obtained from surveillance data. We estimated the relative risk (RR) of acquiring KFD associated with vaccination and then calculated VE as follows: $VE = 1 - RR$.

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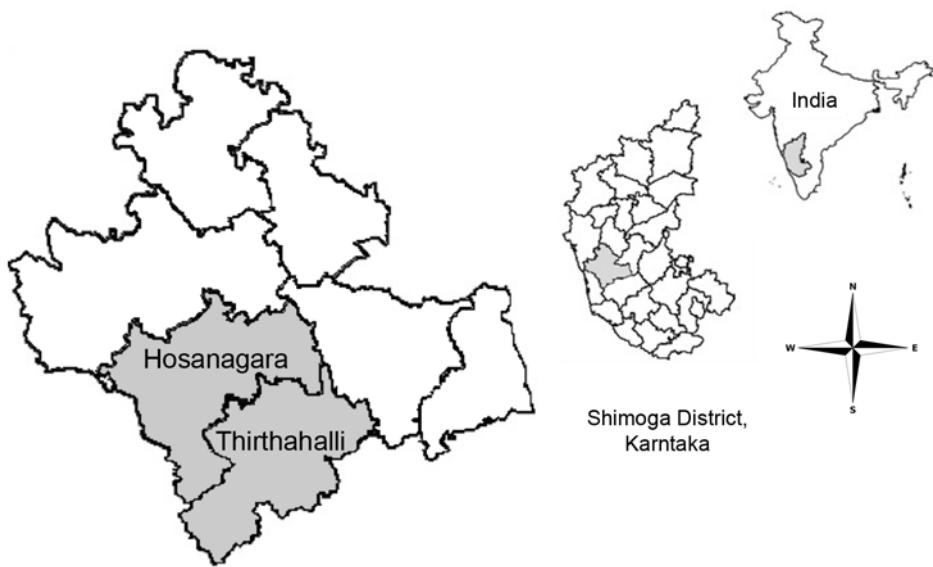


Figure 1. Map of Shimoga District, Karnataka State, India, showing the location of Thirthahalli and Hosanagara Taluks, which were affected by an outbreak of Kyasanur Forest disease virus during December 31, 2013–April 7, 2014, and other taluks within the state. The smaller inset maps show, respectively, the location of Shimoga District within Karnataka State and the location of Karnataka State within India.

During December 31, 2013–April 7, 2014, facility-based surveillance identified 246 suspected cases of KFD; 106 (43.1%) patients were positive for KFDV (78 by RT-PCR, 28 by IgM ELISA); 1 case-patient was also positive for dengue-specific IgM. The laboratory results of suspected case-patients screened up to March 2014 are available elsewhere (6).

Of the 106 case-patients, 102 were from 41 villages in Thirthahalli Taluk (cumulative population 7,317), and 4 were from 3 villages (cumulative population 559) in Hosanagara Taluk (also in Shimoga District) (Figure 1). The overall attack rate was 13.5 cases/1,000 persons. In affected villages, cases were reported from all age groups; rates were highest among persons ≥ 15 years of age and among male residents (Table). Eighteen (16.9%) case-patients were in age groups not targeted for vaccination: 8 were < 7 and 10 were > 65 years of age. The cases began occurring during the last week of December 2013, peaked during February–March 2014, and then declined gradually (Figure 2).

Of the 106 case-patients, 95 (89.6%) reported that they had not been vaccinated and 11 (10.4%) reported being vaccinated (1 received 2 primary and 1 booster dose, 5 received 1 dose, and 5 received 2 doses). Ninety-one case-patients were from villages beyond a 5-km radius of an area with KFD activity in 2013 (i.e., outside an area targeted for vaccination in 2013).

During 2013 (before the outbreak), a total of 19,854 persons 7–65 years of age from Thirthahalli and Hosanagara Taluks had been targeted for KFD vaccination. The coverage of first, second, and booster doses was 23.4%, 15.4%, and 27.3%, respectively.

We included 176 KFD-vaccinated persons (26 received 1 dose, 150 received 2 doses) and 350 unvaccinated

persons in the retrospective cohort study. The vaccinated and unvaccinated persons did not differ with respect to age, sex, or occupation (data not shown). Eight laboratory-confirmed KFD case-patients were reported from this cohort (7 were unvaccinated, 1 had received 2 doses of vaccine). The relative risk associated with 1 and 2 doses of vaccine was 0.96 (95% CI 0.06%–16.5%) and 0.33 (95% CI 0.04%–2.69%), respectively. The VE of 1 and 2 doses of vaccine was 4% (0%–96%) and 67% (0%–96%), respectively.

Conclusions

The findings of our investigation highlighted 4 concerns regarding KFD vaccination strategy practiced in the region. First, vaccine coverage in villages selected for vaccination in 2013 was low. Earlier studies also have shown that nearly half of the eligible population in the targeted villages was not vaccinated (4). These findings indicate low acceptance of KFD vaccine, possibly because of vaccine-associated adverse effects and the need for multiple doses. Second, the observed VE was lower than that reported in a previous study (1 dose, 79%; 2 doses, 94%) (7). However,

Table. Distribution of patients with laboratory-confirmed Kyasanur Forest disease, by age and sex, Shimoga District, Karnataka State, India, December 31, 2013–April 7, 2014

Characteristic	No. persons infected/no. total in affected villages	Attack rate/1,000 population
Age group, y		
0–4	3/677	4.4
5–14	15/1,347	11.1
15–59	74/5,190	14.3
≥ 60	14/662	21.1
Sex		
M	62/4,001	15.5
F	44/3,875	11.4
Total	106/7,876	13.5

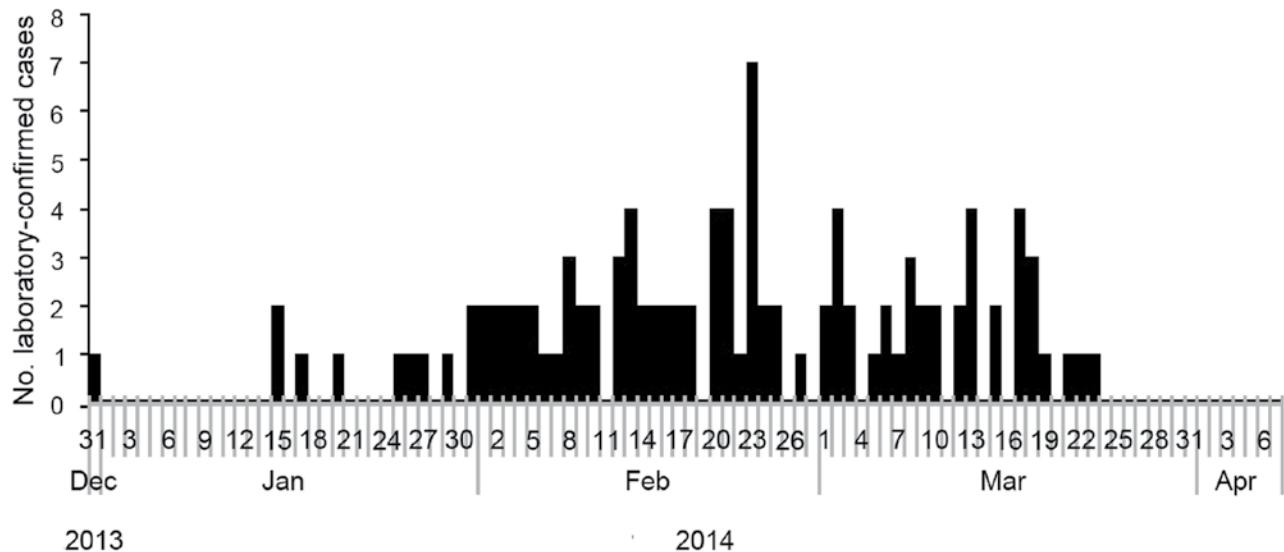


Figure 2. Distribution of 106 laboratory-confirmed Kyasanur Forest disease cases by date of symptom onset, Shimoga District, Karnataka State, India, December 2013–April 2014.

other recent reports also indicate lower VE (2,4,8). Third, the occurrence of cases in areas >5 km away from villages vaccinated in the previous year suggests that targeting vaccination to areas within a 5-km radius of reported KFDV activity may not be effective in preventing KFDV transmission outside the vaccinated areas. KFDV is primarily transmitted by the bite of infected ticks, and it is spread by the movement of monkeys that carry infected ticks; thus, vaccinating around zones with reported KFD activity is unlikely to prevent spread of the virus. Fourth, $\approx 17\%$ of the patients in our study with laboratory-confirmed KFDV infection were <7 or >65 years of age, and persons of these ages are not administered KFD vaccine, probably because lower attack rates were observed among these age groups in earlier outbreaks (7,9,10).

Our study had 2 limitations. First, the facility-based surveillance relied on passive detection of case-patients seeking care from the selected health facilities. The number of case-patients detected is influenced by the health-seeking behavior of the community and by the severity of illness. Second, although VE was found to be low, the cohort study had low power (22.3%); hence, the findings of low effectiveness must be interpreted with caution.

To control KFDV, systematic efforts are needed to improve the current vaccine and vaccine coverage. Current vaccination strategies should be reviewed and the reasons for low VE should be evaluated.

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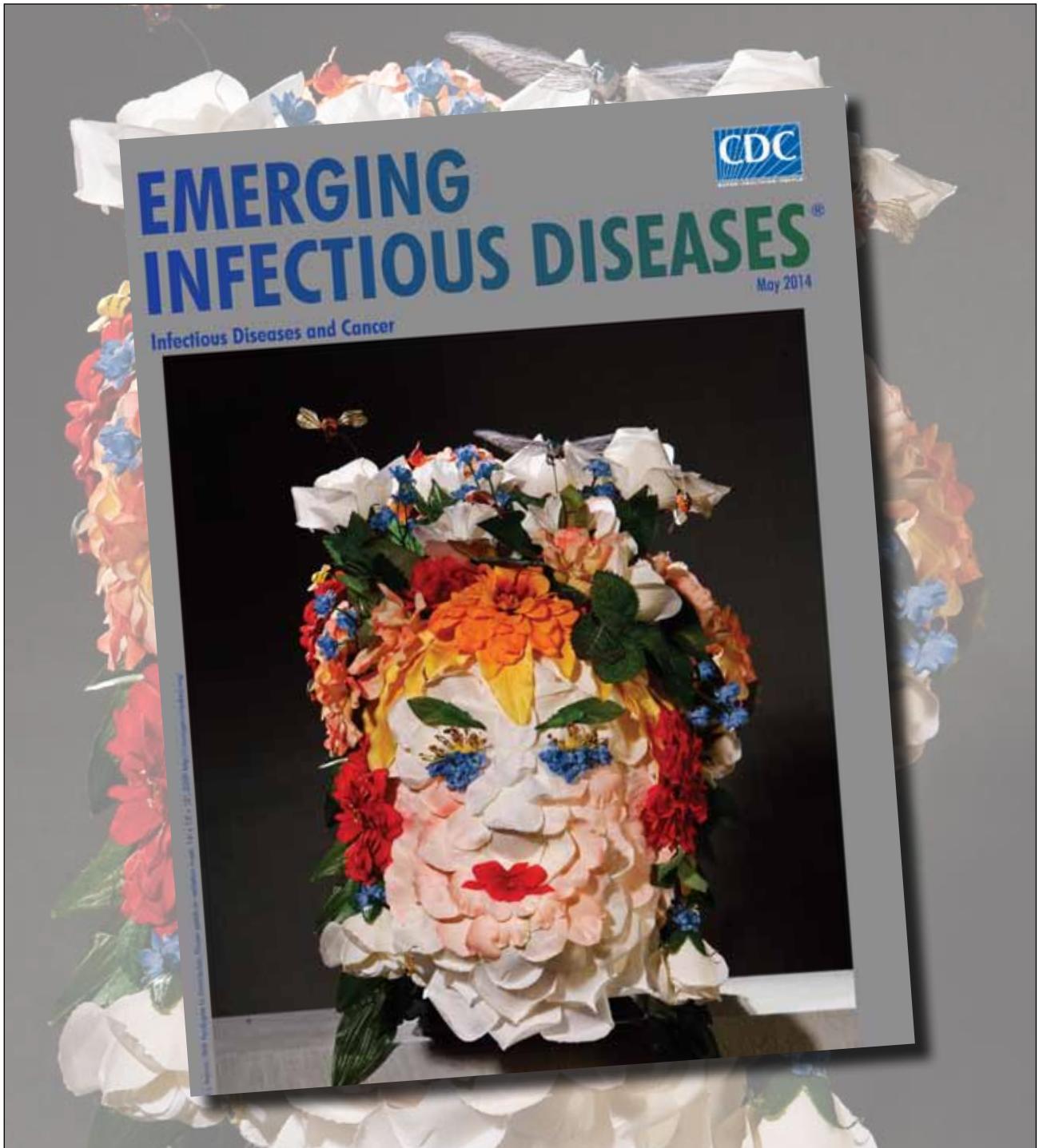
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Hospital-Associated Transmission of *Brucella melitensis* outside the Laboratory¹

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Brucella melitensis was identified in an aspirate obtained from a patient's hip joint during a procedure at a hospital in Canada. We conducted an investigation into possible exposures among hospital workers; 1 worker who assisted with the procedure tested positive for *B. melitensis*. Aerosol-generating procedures performed outside the laboratory may facilitate transmission of this bacterium.

Brucellosis is the most common laboratory-acquired infection (1,2), and laboratory acquisition has been estimated to account for up to 2% of all *Brucella* infections (3). Infection rates among laboratory workers after exposure to *Brucella* spp. have been reported to be as high as 30% (4,5), although recent investigations have described lower attack rates (0–3.8%) (6–9). This difference may be the result of a broader definition of exposure, improved laboratory safety standards, and prompt administration of antimicrobial prophylaxis. Even so, laboratory personnel have experienced severe brucellosis manifestations such as osteomyelitis, meningitis, and death (9). Therefore, manipulation of *Brucella* isolates should occur under Biosafety Level 3 conditions. However, this practice is challenging to implement in developing countries because of lack of resources and high incidence of infection and in industrialized countries because of a low clinical suspicion for brucellosis. In response to the ongoing occurrence of laboratory exposures, the Centers for Disease Control and Prevention (CDC) issued guidelines for the identification and management of laboratory workers potentially exposed to *Brucella* spp., including recommendations for prophylaxis (9,10).

The Study

In July 2012, aspiration of the hip joint of a patient with suspected prosthetic hip infection was performed in the

interventional radiology department (normal pressure, 9 air exchanges/hour) at St. Michael's Hospital in Toronto, Ontario, Canada. Personnel wore gloves but not masks or facial protection. Straw-colored synovial fluid was aspirated into a sterile container. At the time of the procedure, *Brucella* infection was not suspected, although retrospective review showed that the patient had risk factors, including regular travel to India (most recent trip 2 months before the aspirate sample was taken) and consumption of unpasteurized buffalo milk in India. Because the patient was lost to follow-up, we were unable to obtain informed consent for a detailed case description.

The synovial fluid culture was sent to the microbiology laboratory; documentation did not indicate that *Brucella* spp. was a possible etiologic agent. All microbiology specimens at the laboratory are initially processed under a class II biological safety cabinet. Initial Gram stain testing of the sample showed polymorphonuclear cells but no bacteria. On day 3, scant growth of small white colonies was observed on sheep's blood agar and chocolate agar but not on MacConkey agar; Gram stain testing showed gram-negative coccobacilli. The organism was positive for oxidase and catalase, but testing with VITEK2 (bioMérieux, Marcy l'Etoile, France) did not identify the organism. By that time, the sample had been on an open bench for 6 days, and the sample was then referred to the provincial reference laboratory. There, the organism was identified as *B. melitensis*—18 days after the specimen was initially obtained and 10 days after it was sent to the reference laboratory.

After *B. melitensis* was identified, we initiated an investigation to identify laboratory personnel who may have been exposed to or infected with the bacterium. CDC recommendations from 2008 were used to classify laboratory personnel into high- and low-risk categories and to guide prophylaxis and follow-up (9). Although the CDC guidance did not address the management of exposure among nonlaboratory health care workers (HCWs), we considered HCWs who were in the procedure room during the aspiration to be at high risk. A total of 12 persons were identified as high risk (10 from the laboratory, 2 from radiology); 20 laboratory personnel were identified as low risk.

All HCWs classified as high risk completed prophylaxis with 3 weeks of doxycycline (100 mg orally 2×/d)

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and rifampin (600 mg orally 2×/d). Serial serologic testing at baseline, 2, 4, 6, and 24 weeks after *B. melitensis* was identified was recommended for all 32 exposed HCWs. Serologic testing for IgG was performed at the provincial reference laboratory by using an in-house serum tube agglutination test. Thirty HCWs (10 high-risk and 20 low-risk) completed the serial serologic testing (Table); however, the baseline serologic tests were obtained ≈3 weeks after the initial exposure because of the delay in identifying *Brucella* in the culture of the aspirate.

A radiology technician classified as high-risk (held the specimen container during the procedure and assisted with injecting the synovial fluid from a syringe into a container) had serologic test results positive for *Brucella*. Her initial titer, obtained 3 weeks after exposure, was 1:160. She began antimicrobial drug prophylaxis 2 days after the initial sample was sent to the laboratory; on the basis of the positive result, drug therapy as noted above was extended to 6 weeks. A repeat titer 8 weeks after exposure was 1:320, and further titers performed at 24 weeks–20 months after exposure remained elevated ($\geq 1:160$). Two years postexposure, she remains asymptomatic. She consented to have her epidemiologic and clinical data used in this report.

All HCWs with negative baseline results remained negative for the duration of follow-up. Two staff members who had indeterminate titers had no known risk factors for *Brucella* infection other than the laboratory exposure and remained asymptomatic for 24 weeks with stable titers.

Conclusions

Brucella is a well-recognized cause of occupationally acquired infection among microbiology laboratory staff. However, *Brucella* infection was not suspected in this case because of its rarity both in Canada and as an etiologic agent in prosthetic joint infections (11). The delay in organism identification and completion of aerosolizing procedures (e.g., catalase test) on an open laboratory bench increased the risk for exposure among hospital workers. Prompt identification and prophylactic treatment of high-risk laboratory staff members prevented clinical disease and seroconversion.

Previous descriptions of *Brucella* outbreaks have focused on laboratory-associated exposures (2,6,7,9,10). Synovial fluid is considered a low-risk specimen for *Brucella* exposure because the number of organisms is low and the risk for transmission is reported to be minimal compared with exposure to purified organisms in the laboratory (1). In this instance, however, transmission was theoretically possible: aspiration of the joint and forceful ejection of the synovial fluid from a syringe into a sterile container could result in aerosolization.

Table. Baseline serologic test results for 32 health care workers exposed to *Brucella melitensis* in Toronto, Canada, 2012

Risk and test result categories	Test results, no. workers (titer, if applicable*)
High-risk, radiology, n = 2	
Negative†	1
Indeterminate	0
Positive	1 (1:160)
High-risk, laboratory, n = 10	
Negative	9
Indeterminate	1 (1:80)
Positive	0
Low-risk, laboratory, n = 20	
Negative	18
Indeterminate	1 (1:80)
Positive	0
Refused testing	1

*Negative, $\leq 1:20$; indeterminate, 1:40–1:80; positive $\geq 1:160$.
†Lost to follow-up, but serologic results were negative at 6 weeks after exposure.

The HCW with positive serologic test results was a radiology technician who assisted in the procedure. She was born in Egypt and immigrated to Canada in 2003, but she had no subsequent travel back to Egypt or other *Brucella*-endemic areas and no other risk factors for infection. Her elevated titers might have occurred because of past exposure in a *Brucella*-endemic country, but she had left Egypt 9 years prior, and her titers would be expected to be low, even if she had a distant history of *Brucella* infection. Furthermore, in *Brucella*-endemic countries, serum agglutination titers $\geq 1:160$ are considered positive (12). Her titers were elevated on subsequent testing to as high as 1:320, but the serologic response may have been blunted by prompt antimicrobial drug treatment.

Hospital-associated transmission of *Brucella* outside of the laboratory setting may represent a rare occurrence. Two cases of transmission from mother/child to obstetrician have been described (13,14). Similar criteria for investigating exposures were used in a case of a *B. abortus* hip infection in the operating room but did not identify any transmissions (15). However, cases identified in *Brucella*-endemic areas may have been attributed to community, rather than occupational, exposure.

On the basis of our findings, we recommend that HCWs performing aspiration or other aerosolizing procedures on patients with known or suspected *Brucella* infection should use fit-tested N95 respirators and other appropriate personal protective equipment, including gloves, gown, and facial protection. If exposure occurs without the use of appropriate protective equipment, monitoring and serologic follow-up should be initiated, as well as possible prophylaxis for those at highest risk (e.g., performing procedure, holding specimen). Follow-up is critical in non-*Brucella*-endemic areas because the incubation period is prolonged, clinical suspicion may be low, and the potential for delayed diagnosis in the event of illness is high.

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Dr. Lowe was a resident in medical microbiology at the University of Toronto at the time of the study. He is currently a staff medical microbiologist and infection prevention and control physician at Providence Health Care in Vancouver, British Columbia, Canada. His research interests are focused on identifying optimal methods for infection control of multidrug-resistant gram-negative organisms and hospital-acquired infections.

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Hare-to-Human Transmission of *Francisella tularensis* subsp. *holarctica*, Germany

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In November 2012, a group of 7 persons who participated in a hare hunt in North Rhine-Westphalia, Germany, acquired tularemia. Two *F. tularensis* subsp. *holarctica* isolates were cultivated from human and hare biopsy material. Both isolates belonged to the FTN002–00 genetic subclade (derived for single nucleotide polymorphisms B.10 and B.18), thus indicating likely hare-to-human transmission.

Tularemia is a zoonotic disease caused by the gram-negative bacterium *Francisella tularensis* (1). Currently, there are 4 validly published subspecies. *F. tularensis* subsp. *tularensis* is the most virulent subspecies and occurs only in North America. *F. tularensis* subsp. *holarctica* is less virulent and occurs throughout the Northern hemisphere. *F. tularensis* subsp. *mediasiatica* was isolated in central Asia, and *F. tularensis* subsp. *novicida*, which has low virulence in humans, seems to be distributed globally (2).

Various PCR-based assays have been established for the detection of *F. tularensis* or for the diagnosis of tularemia. An accurate population structure has been defined by using single nucleotide polymorphisms (SNPs) and insertion/deletion mutations (INDELs) with potential canonical properties. Currently, this population is divided into 4 major genetic clades: B.4, B.6, B.12, and B.16 (3–6). The taxonomic nomenclature of major clades in *F. tularensis* subsp. *holarctica* is based on clade-specific canonical SNP markers (3,4). In Europe, the strains of clades B.12 and B.6 dominate (6). The latter is found particularly in large areas in northern, western, and central Europe, including Germany (5–9).

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The Study

On November 2, 2012, 15 European brown hares (*Lepus europaeus*) were shot during a hunt in Rütten-Meiste, district Soest in the federal state of North Rhine-Westphalia, Germany (Figure). The animals seemed healthy and showed normal escape behavior. Upon inspection, the animals that had been shot showed no signs of disease. Consequently, all animals were skinned, eviscerated, and dissected. Portioning of the hares was done 2 days later. Within a few days, 7 healthy persons who had contact with the hare carcasses showed varied symptoms of illness. Tularemia was suspected because of the signs and symptoms in combination with exposure in a tularemia-endemic area. Exposure, clinical symptoms, and time of onset of symptoms of all patients (A to G) are described in the Table. All patients were treated successfully with doxycycline.

Human serum samples were collected about 5 weeks after infection from patients C and E and pleural fluid was obtained from patient E. On day 2 of incubation, the human isolate (12T0062) showed small pale-white to gray colonies on Columbia blood agar and chocolate agar, whereas no growth occurred on MacConkey agar plates. Ten organ specimens (from aorta, back and thigh muscles, lymph nodes, spinal cord) from 4 of the hares handled by the patients were tested for *Francisella* spp. in the National Reference Laboratory for Tularemia at the Friedrich-Loeffler-Institut in Jena, Germany. From all hare organs, only a single *Francisella* sp. was isolated from a spinal cord sample (13T0009) on cysteine heart agar Becton Dickinson GmbH, Heidelberg, Germany), which contains antibiotics. The strains were susceptible to erythromycin with inhibition zones between 22 and 24 mm corresponding to biovar I. Details of further methods that have been applied in the study are shown in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/13-1837-Techapp1.pdf>).

Both samples were identified as *F. tularensis* subsp. *holarctica* of clade B.6. For B.19, the SNP was C; results of Ftind33 and Ftind38 assays were IN, and the result of the Ftind49 assay was DEL. Both samples had T for SNP B.7, G for B.10 and T for B.18. Therefore, the strains were considered derived from SNPs B.10 and B.18. Blood serum samples of patients C and E were positive for *F. tularensis* with very high values of the optical density in the ELISA, 2.886 and 3.121, respectively.



Figure. Area of Germany where hares were hunted on November 2, 2012: R then-Meiste (black star; latitude 51.512890, longitude 8.487493, altitude 380 m), Soest district (white) of the federal state of North Rhine-Westphalia (dark gray).

Conclusions

The re-emergence of tularemia in Germany has been described in previous studies (10). Infected hares are believed to be the sources of most cases of tularemia in Germany. However, to our knowledge, route of transmission has not been demonstrated by isolation and genotyping of the pathogen from the suspected source and the patient (11–13). In this study, we therefore described not only the clinical and epidemiologic data and the laboratory diagnostic findings for determining tularemia, but also the results of genotyping the *Francisella* spp. isolated from epidemiologically linked hares and humans.

All 7 infected persons (A to G) in this outbreak showed influenza-like symptoms of varying intensity (Table), but symptoms were also related to the route of infection. The 5 patients (A to E) who had fever as well as respiratory and topical symptoms were exposed to aerosols and had direct skin contact when skinning and processing hare carcasses. The 2 patients (F and G) who portioned the meat had lesions on their hands, enlarged lymph nodes, and fever.

The isolated *F. tularensis* subsp. *holarctica* strains were susceptible to erythromycin and thus belong to the *F. tularensis* subsp. *holarctica* biovar I group. Because of the inability of the duplex PCR assay to distinguish between *F. tularensis* subsp. *holarctica* strains (8), we performed a combined SNP and INDEL analysis using real-time PCR. Here, we were able to isolate *F. tularensis* subsp. *holarctica* biovar I strains from a hare and a human; both isolates could be assigned to the genetic clade B.6 in the first order of discrimination [B.19(C), Ftind33(IN), Ftind38(IN), and Ftind49(DEL)]. The isolates also showed an identical genotyping profile for B.7(T), B.10(G), B.18(T) in the second order of discrimination, which corresponds with a previously described subclade represented by the strain FTNF002–00 that was isolated from a patient from France who had bacteremia (3,4). Thus, the genetic subtyping results are consistent with the proposed transmission route of the epidemiologically linked (hare–human transmission) *F. tularensis* subsp., since both belonged to the same genetic subclade.

The current phylogeography of *F. tularensis* subsp. *holarctica* revealed that 2 major groups of virulent strains exist in Europe (5). In the western European countries of Spain, France, Switzerland, and Italy, strains of the FTNF002–00 group dominate, whereas strains of clade B.12 seem to predominate in eastern and northern Europe as reported from Austria, Czech Republic, Finland, Georgia, Hungary, Romania, Russia, Slovakia, Sweden, and Ukraine (4,5,8,13). Vogler et al. (4) suggest that it is likely that the spread of strains in subclade FTNF002–00 throughout France and the Iberian Peninsula was a very recent event. In Germany, isolates of both groups have been identified and a sharp dividing line in terms of occurrence of the clades B.12 and B.6 from the northwest to the southeast of the country has been shown (8). The reasons for this are not known; possible causes could be environmental and epidemiologic differences. Alternately, a mixture of both genetic clades and biovars have been reported in Bulgaria, Kazakhstan, Norway, Russia, and Sweden (7,14,15).

The genome of *F. tularensis* subsp. *holarctica* is highly conserved and strains can hardly be discriminated. Therefore, the discriminatory power of the applied assays is limited and other field isolates from this area may show identical characteristics (H. Tomaso, unpub. data). For epidemiologic and forensic purposes, whole-genome sequencing of a multitude of strains from well-documented outbreaks and the surrounding areas should be performed to clarify and possibly quantify the genetic changes that can finally confirm or rule out the route of transmission.

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Table. Exposure, clinical presentation, and time of onset of clinical symptoms of tularemia in patients, Germany, 2012

Patient	Exposure type	Clinical symptoms	Symptom onset
A	Skinning	Fever, cold, cough	Nov 12
B	Skinning	Chills, fever, joint pain	Nov
C	Dissection	Fever, nodes and skin ulcers	Nov
D	Cut up carcasses	Fever, cold, cough, joint pain, convulsions	Nov
E	Cut up carcasses	Cough, pleural effusion, weight loss	Unknown
F	Portioning of hares	Fever, skin ulcers, lymphadenopathy	Unknown
G	Portioning of hares	Fever, skin ulcers, weight loss	Unknown

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Dr. Otto is the head of the working group, Noro- and Rotaviruses, and an employee of the National Reference Laboratory of Tularemia at the Federal Institute of Bacterial Infections and Zoonoses of the Federal Research Institute of Animal Health, Friedrich Loeffler Institut, Jena, Germany. His interests are focused on the development of diagnostic methods, the incidence and epidemiology of *Francisella* spp., and other bacterial zoonotic agents in wild animals.

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Streptococcus equi* subsp. *zoepidemicus **Infections Associated with Guinea Pigs**

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Joseph P. Garvin, Susan Brown, Lauren Turner,
Angela Fritzingler, Robert E. Gertz, Jr.,
Julia M. Murphy, Marshall Vogt,
and Bernard Beall**

Streptococcus equi subsp. *zoepidemicus* is a known zoonotic pathogen. In this public health investigation conducted in Virginia, USA, in 2013, we identified a probable family cluster of *S. zoepidemicus* cases linked epidemiologically and genetically to infected guinea pigs. *S. zoepidemicus* infections should be considered in patients who have severe clinical illness and report guinea pig exposure.

Streptococcus equi subsp. *zoepidemicus* is a facultative pathogen affecting animals and humans. Infections have occurred in horses, pigs, ruminants, guinea pigs, monkeys, cats, and dogs (1,2). Zoonotic transmission of *S. zoepidemicus* is rare and is usually associated with drinking unpasteurized milk or through contact with horses by persons who usually have underlying health conditions (1–3). Few if any human case-patients with *S. zoepidemicus* infection have documented guinea pig exposure even though *S. zoepidemicus* infections have been described in guinea pigs since 1907 (4). This case report describes 1 probable and 1 confirmed human case of severe *S. zoepidemicus* infection and the laboratory methods used to link human and guinea pig isolates.

Case Reports

An adult man (patient 1) from northern Virginia, USA, arrived at a hospital in late February 2013 with influenza-like symptoms, worsening bilateral thigh pain and stiffness, nausea, shivering, fatigue, diarrhea, sweating, and headache. Past medical history included exercise-induced asthma, nephrolithiasis, and slightly elevated liver function test results for “a couple of years,” as stated in the medical record. Initial physical examination of

the patient revealed mild scleral icterus, rhabdomyolysis, and rash on his thighs. The patient’s elevated liver function test results were attributed to the rhabdomyolysis. Shortly after being admitted, the patient experienced acute renal failure, sepsis, pneumonia, and bilateral lower extremity edema, the latter of which was thought to be compartment syndrome. Blood cultures performed at the hospital showed group C streptococcal infection. Because of the patient’s worsening condition, he was transferred to a tertiary care center. At the tertiary care center, the patient was treated for septic shock secondary to rhabdomyolysis, placed on a ventilator several times to treat respiratory failure, and underwent bilateral thigh fasciotomy and debridement several times to treat necrotizing fasciitis. Wound cultures identified *S. equi* as the causative agent; a subspecies was not specified, although *zoepidemicus* was likely because it is the only zoonotic subspecies of *S. equi*. After treatment at the tertiary facility for several months, the patient was discharged to a rehabilitation hospital for another month.

An elderly man from central Virginia (patient 2) who was related to patient 1 was admitted to a hospital, 1 week after patient 1 was hospitalized, with nausea, vomiting, chills, difficulty breathing, weakness, abdominal and chest pain, and icterus. The medical history of patient 2 included smoking, oral cancer, myocardial infarction, hypertension, hyperlipidemia, and coronary artery disease. By the second day in the hospital, patient 2 experienced acute hypoxia and respiratory failure; pneumonia in the right lower lobe was diagnosed. He also had hypotension secondary to septic shock and multiple organ failure. Group C *Streptococcus* spp. were identified in blood cultures 2 days after hospitalization. Patient 2 was discharged 18 days after hospitalization and was receiving continuous oxygen.

The local health department for the area in which patient 1 resided was contacted by the tertiary care center where he was treated because of the probability that the *S. equi* infection was caused by guinea pig exposure. Questioning of a female relative of patient 1 (relative 1) indicated that patient 1 recently purchased 4 guinea pigs and that 1 had died shortly after purchase. During the interview with relative 1, it was learned that patient 2 was hospitalized. After another female relative (relative 2) was interviewed, it was learned that patient 2 had cleaned the guinea pigs’ enclosure 2 days before his illness. The Virginia Department of Health (VDH) requested isolates from both patients to be forwarded to the Division of Consolidated Laboratory Services (DCLS), the state

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public health laboratory. Only an isolate from patient 2 was available and forwarded. VDH representatives also discussed the likelihood of disease in patients 1 and 2 being caused by the guinea pigs with the relatives. Relative 2, who was caring for the guinea pigs at the time, relinquished the 3 remaining guinea pigs which were sent to a Virginia Department of Agriculture and Consumer Services laboratory for euthanasia and microbiological testing.

Using API 20 Strep strips (bioMérieux, Inc., Durham, NC, USA), laboratory personnel identified *S. zooepidemicus* in isolates from 5 specimens (1 lymph node, 2 conjunctival swabs, and 2 nasal passage swabs) collected from 2 of the 3 guinea pigs. No apparent lesions were noted in the guinea pigs upon euthanasia and sample collection. Three isolates from the guinea pigs, 1 from each site, were forwarded to DCLS.

At DCLS, patient 2's isolate was also identified as *S. zooepidemicus* by API 20 Strep strips. DCLS staff performed pulsed-field gel electrophoresis (PFGE) testing on all 4 isolates. Briefly, plugs were prepared by incubating washed cells in Tris-EDTA buffer solution (pH 8.0) with 10 mL lysozyme (10 mg/mL) before mixing with 1.5% molecular grade agarose and 0.17 mg/mL proteinase K (Roche, Mannheim, Germany) (5). Cells were lysed in extracellular buffer with 1 mg/mL lysozyme and 0.3 mg/mL proteinase K, for 1.5 h at 37°C (6). After a MilliQ water wash and 3 Tris-EDTA buffer washes, DNA was digested with 100 U *Sma*I and 100 U *Apa*I (both from New England Biolabs, Inc., Ipswich, MA, USA) and separated on a 1% agarose gel at 6 V/min, 14°C for 17.5–18 h in 0.5× TBE buffer by using a CHEF Mapper XRS electrophoresis system (BioRad, Hercules, CA, USA). The running parameters were as follows: for *Sma*I: initial pulse, 5.3 sec; final pulse, 34.9 sec; *Apa*I: initial pulse, 2.0 sec; final pulse, 25 sec. The gels were visualized by using ImageLab (BioRad) software with a ChemiDoc XR+ (BioRad). An *Enterococcus faecalis* control strain was used for image normalization during DNA fingerprint pattern analysis with BIONUMERICS 5.1 software (Applied Maths, Austin, TX, USA). The banding patterns were compared by using Dice coefficients with a

1.0%–1.5% band tolerance. PFGE results indicated that all 4 isolates were indistinguishable by comparison with the *Sma*I and *Apa*I enzymes (Figure).

Multilocus sequence typing was performed as described (<http://pubmlst.org/szooepidemicus/>) based on the allelic profile of 7 housekeeping gene fragments. The isolates from patient 2 and the 2 guinea pigs shared the same 6-locus profile of 370- to 459-bp housekeeping gene sequences. The 6-locus profile, *arcC27*, *nrdE19*, *spi45*, *tdk1*, *tpi34*, *yqiL46*, was found in each of the 4 isolates rather than a normally obtained 7-locus profile because the *proS* locus did not amplify. This 6-locus profile was unique: its closest match in the *S. zooepidemicus* database was ST194 (*arcC27*, *nrdE3*, *proS1*, *spi45*, *tdk1*, *tpi34*, *yqiL46*); both had 5 of the 6 gene-fragment sequences (Table). ST194 is recorded in the *S. zooepidemicus* database from 2 human blood isolates recovered during 2001. We subsequently found that ST194 is also shared by ATCC *S. zooepidemicus* strain 35246, which was isolated from a diseased pig in China (7).

In addition, the Centers for Disease Control and Prevention laboratory staff sequenced the so-called M-like protein gene (*szp*) of these isolates, using previously described sequencing and amplification primers (8). The 1,128-bp structural gene sequence (GenBank accession no. KF722996) was found to be identical for the 4 isolates and closely matched the 1,140-bp M-like gene (*szp*) from the ATCC 35246 strain. The only difference was deletion of 1 of 10 consecutive 4-codon repeats (PKPE, codons 277–280) (9).

Conclusions

S. zooepidemicus infection should be considered in patients who have purulent wounds or systemic symptoms of infection who have had known contact with guinea pigs or their environment. Likewise, patients whose specimen cultures reveal *S. equi* or further test results show *S. zooepidemicus* should be questioned about guinea pig exposure as well as exposure to other animals associated with this pathogen: horses, pigs, ruminants, monkeys, cats, and dogs.

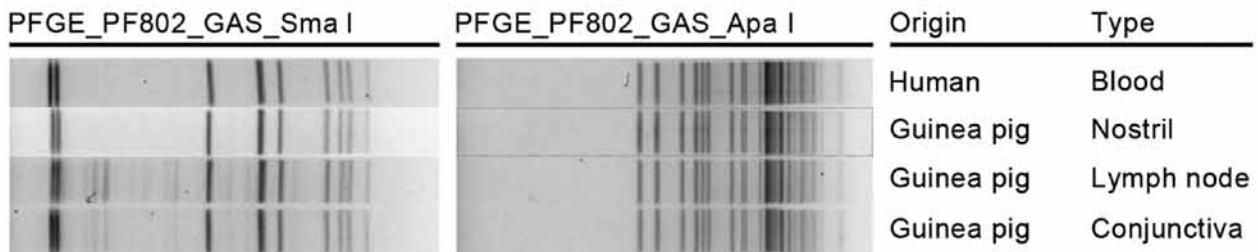


Figure. Pulsed-field gel electrophoresis (PFGE) patterns for 4 *Streptococcus equi* subsp. *zooepidemicus* isolates from 1 person and 3 guinea pigs submitted to the Division of Consolidated Laboratory Services, Virginia, USA. Patterns indicate that all 4 isolates were indistinguishable by the *Sma*I and *Apa*I enzymes. Specimen origin and type are indicated.

Table. Closest species matches to multilocus sequence typing alleles of study *Streptococcus equi* subsp. *zooepidemicus* isolates

Allele*	Length, bp	Closest GenBank overall species match (% identity/overlap)†	Next best GenBank matches (% identity/overlap)†
<i>arcC27</i>	437	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, ATCC35246 (100%/437 bp); H70 (99%/437 bp); MHCS10565 (97%/437 bp)	<i>S. equi</i> subsp. <i>equi</i> strain 4047 (97%/437 bp)
<i>nrdE19</i>	448	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, ATCC35246, MGCS10565 (99%/448 bp); H70 (97%/448 bp)	<i>S. equi</i> subsp. <i>equi</i> strain 4047 (94%/448 bp); <i>S. thermophilus</i> ASCC1275, JIM8232, ND03, 5 others (80%/447 bp)
<i>spi45</i>	459	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, ATCC35246, 4047 (100%/459 bp); MGCS10565 (99%/459 bp); H70 (94%/459 bp)	<i>S. dysgalactiae</i> subsp. <i>equisimilis</i> strains ATCC12394, 167, AC2713, RE378, GGS124 (72%/447 bp)
<i>tdk1</i>	370	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, 636199–1A, 65843, ATCC35246, H70 (100%/370 bp); 15 others (99%/370 bp)	<i>S. equi</i> subsp. <i>equi</i> strain 4047 (99%/370bp); <i>S. equi</i> subsp. <i>zooepidemicus</i> 10 strains (96%–98%/369 bp)
<i>tpi34</i>	424	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, 14580–1A, 44464, ATCC35246 (100%/424bp); 22 others (99%/424 bp); 27 others (95%–97%/424 bp)	<i>S. equi</i> subsp. <i>equi</i> strain 4047 (97%/424 bp); <i>S. pyogenes</i> 20 strains (82%/422 bp)
<i>yqiL46</i>	396	<i>S. equi</i> subsp. <i>zooepidemicus</i> strains CY, ATCC35246 (100%/396 bp); >30 strains (99%/396 bp); >20 strains (95%–98%/396 bp)	<i>S. equi</i> subsp. <i>equi</i> strain 4047 (97%/424 bp); <i>S. pyogenes</i> MGAS6180 (70%/422 bp)

*All 4 isolates shared the identical *arcC27*, *nrdE19*, *spi45*, *tdk1*, *tpi34*, *yqiL46* alleles; sequences provided at <http://pubmlst.org/zooepidemicus>.

†Searches performed at <http://blast.ncbi.nlm.nih.gov/Blast.cgi>

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Association of Melioidosis Incidence with Rainfall and Humidity, Singapore, 2003–2012

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Kee Tai Goh, Sharada Ravikumar, Mar Soe Win,
Gladys Tan, Alex Richard Cook, Dale Fisher,
and Louis Yi Ann Chai

Soil has been considered the natural reservoir for the bacterium *Burkholderia pseudomallei*, which causes melioidosis. We examined 550 melioidosis cases that occurred during a 10-year period in the highly urbanized city of Singapore, where soil exposure is rare, and found that rainfall and humidity levels were associated with disease incidence.

The gram-negative, saprophytic bacillus *Burkholderia pseudomallei*, which causes melioidosis, is endemic in northern Australia and Southeast Asia countries such as Thailand, Malaysia, and Singapore (1). Soil has traditionally been described as the natural reservoir of *B. pseudomallei* (hence the synonym “soil bacteria”) (2,3). Symptoms and signs of melioidosis can be mild, but severe manifestations such as bacteremia, organ abscesses, and severe pneumonia can lead to high death rates (4).

Two reports from northern Australia and northeastern Thailand, both conducted in predominantly rural areas, found an increase in melioidosis cases after heavy rainfall or extreme weather events such as tropical storms or monsoons (5,6). In Singapore, however, the occurrence of melioidosis in association with climatic variations has not been extensively studied. Geographically, Singapore has an urbanization rate of 100%, in contrast to northeastern Thailand (34%) and northern Australia (55%) (7,8). Because soil is the presumptive reservoir for *B. pseudomallei*, exposure may be less common in an extensively developed, urban setting such as Singapore, but other environmental factors, such as humidity and temperature, might also influence the pathogenicity of *B. pseudomallei*.

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We investigated 550 cases of melioidosis that occurred during a 10-year period in Singapore to determine if climatic factors might be related to the epidemiology of this disease in an urban setting.

The Study

Melioidosis is a notifiable infectious disease in Singapore. Clinical and laboratory criteria for notification according to the Ministry of Health, Singapore, are a positive culture of *B. pseudomallei* (which accounts for 96.5% of notified cases) or strongly positive serologic test results combined with appropriate clinical manifestations (9). For our investigation, we obtained weekly melioidosis case numbers compiled on the basis of disease onset dates during 2003–2012 from the Ministry of Health, Singapore (10). Data on patient sex, age, and race were also included. Monthly and weekly rainfall, humidity, and temperature humidity data were obtained from the Singapore Meteorological Service, Ministry of Environment and Water Resources, and from Weather Underground (<http://www.wunderground.com>).

To assess the correlation between the incidence of melioidosis and rainfall, humidity, and temperature, we built regression models that used a quasi-Poisson distribution for the number of cases; Poisson and quasi-Poisson models are suitable for count data, of which the quasi-Poisson requires fewer assumptions. These models analyzed data at monthly and weekly intervals throughout the study period. Wald tests (i.e., the standard statistical test for regression models with nonnormal distributions, such as logistic and Poisson regressions) were conducted for various time lags after illness onset. Statistical significance was set at $p < 0.05$. Statistical analyses were performed by using R Statistical Software version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria).

During the 10-year study period, 550 cases of melioidosis (range 31–96 cases per year) were notified in Singapore (Table 1). Of the patients, 84.1% were male, a higher percentage than found in previous studies in Thailand (57% [11]) and 69% for northern Australia (69% [1]). Mean patient age was 51.3 years.

The overall incidence of melioidosis in Singapore during the study period was 1.1 cases per 100,000 population. Disease incidence was highest among Malays and Indians (2.5 and 2.2 per 100,000 population, respectively). The mortality rate from the disease was 19.0%, similar to that

Table 1. Melioidosis case distribution by year, Singapore, 2003–2012*

Category	2003†	2004	2005	2006	2007	2008	2009	2010	2011	2012	Total cases
No. cases	44	96	74	59	57	60	37	58	34	31	550
Mean patient age, y	52.6	51.3	51.2	50.6	56.9	49.6	54.1	55	46.1	45.9	51.3
Patient sex											
F	6	15	9	13	7	9	8	13	6	1	87 (15.9)
M	36	81	65	46	50	51	29	45	28	30	461 (84.1)
Race/ethnicity											
Chinese	27	62	36	35	25	35	19	28	16	11	294 (65.6)
Malay	10	14	20	11	17	14	4	16	8	9	123 (27.5)
Indian	3	11	10	7	14	8	5	2	4	5	69 (15.4)
Others	0	4	2	0	0	3	7	3	4	0	23 (5.1)
Foreigners	2	5	6	6	1	0	2	9	2	6	39 (8.7)
Incidence by race											
Chinese	0.9	2.3	1.3	1.3	0.9	1.3	0.7	1	0.5	0.4	1.1
Malay	2	2.9	4.1	2.2	3.5	2.8	0.8	3.2	1.6	1.8	2.5
Indian	0.7	3.8	3.2	2.2	4.5	2.5	1.5	0.6	1.1	1.4	2.2
Total incidence	1	2.3	1.7	0.7	1.2	1.2	0.7	1.1	0.7	0.6	1.1
Deaths	6 (13.6)	26 (27.1)	12 (16.2)	9 (15.3)	12 (21.1)	12 (20.0)	5 (13.5)	14 (24.1)	6 (17.6)	2 (6.5)	104 (19.0)

*Values are no. (%) except as indicated.

†Data for 2 cases in 2003 were unavailable.

for northern Australia (14%) (4), which likely reflects similar of health care provisions for the 2 cities; in contrast, the mortality rate for Thailand was 43% (11).

During the study period, increased case numbers were generally observed during July–October and in January.

The average total monthly rainfall for the period was 192.5 mm ± 121.6 mm (range 6.3–765.9 mm), and the average humidity and temperature were 83.7 mm ± 2.5% (range 77.3%–88.5%) and 27.7°C ± 0.7°C (range 26.3°C–29.2°C), respectively.

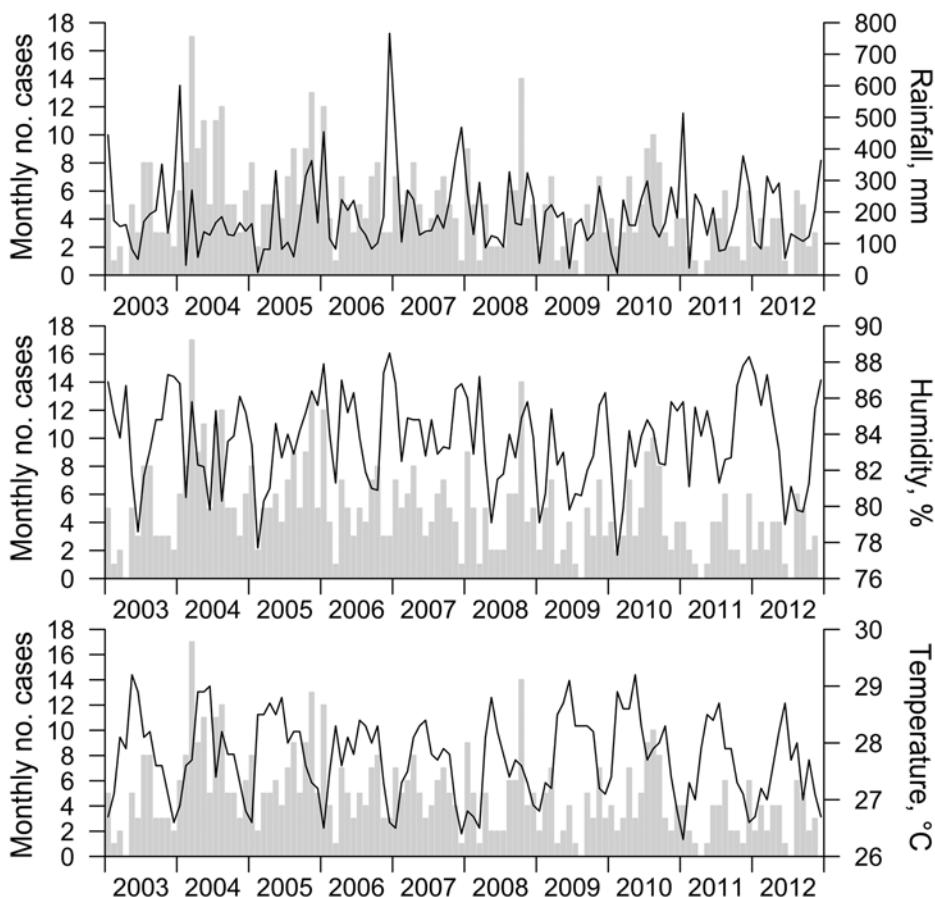


Figure. Total monthly rainfall (mm), average monthly humidity (%), and average monthly temperature (°C) compared with melioidosis case numbers, Singapore, 2003–2012. Cases are listed by date of illness onset.

Table 2. Temporal association of rainfall, humidity, and temperature with melioidosis cases, Singapore, 2003–2012*

Category	No lag		1-week lag		2-week lag	
	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value	Hazard ratio (95% CI)	p value
Rainfall	1.20 (0.868–1.65)	0.274	1.40 (1.03–1.90)	0.0345	1.34 (0.981–1.83)	0.0667
Humidity	1.01 (0.985–1.04)	0.436	1.02 (0.997–1.05)	0.0806	1.03 (1.00–1.05)	0.0382
Temperature	1.01 (0.910–1.13)	0.814	0.949 (0.851–1.06)	0.351	0.928 (0.832–1.03)	0.178

*Association between melioidosis cases and weather conditions during the week of disease onset (no lag), 1 week before disease onset (1-week lag), and 2 weeks before disease onset (2-week lag). Significance is indicated by $p < 0.05$.

The Figure shows a plot of the variations in total rainfall, average humidity, and average temperature by month along with corresponding numbers of melioidosis cases. We found a significant correlation between the number of melioidosis cases and the volume of rainfall in the 1-week period before disease onset, with a hazard ratio (HR) of 1.40 per 100 mm increase in rain (95% CI 1.03–1.90; $p = 0.03$) (Table 2). The humidity level 2 weeks before disease onset was more modestly associated with the number of cases (HR 1.03 per 1% increase in humidity, 95% CI 1.00–1.05; $p = 0.04$), but this value did not have an independent association beyond that of rainfall in multivariable analysis; rainfall and humidity shared a positive correlation at a 1-week lag interval ($R = 0.45$; $p < 0.001$). We found no association between temperature and the number of melioidosis cases.

Conclusions

Soil is considered the natural reservoir of *B. pseudomallei* (2,3), but in the highly urbanized city of Singapore, the likelihood of soil exposure predisposing to infection by *B. pseudomallei* may reasonably be considered to be low. We found a significant correlation of melioidosis cases in Singapore with higher rainfall totals and, to a lesser degree, to higher humidity levels. This finding indicates that water, rather than soil, may be the central vehicle for transmission and acquisition of this disease. Epidemiologic data from rural Thailand and northern Australia (5,6) suggest that incremental volumes of rainfall result in raising the water table on land, which leads to accumulation of *B. pseudomallei* on surface soil, which becomes a reservoir for inhalation of aerosolized bacteria. However, most (82.0%) patients with melioidosis in Singapore did not report occupational or recreational exposure to soil (Communicable Diseases Division, Ministry of Health, Singapore, unpub. data).

We found a 1-week interval between periods of heavy rainfall and increased cases of melioidosis; a comparable study in Australia cited a 14-day lag (5). However, our results are supported by observations from a 6-month epidemiologic investigation conducted in 2004 that described a relationship between the incidence of melioidosis and cumulative rainfall amounts 7 days before onset of illness (12). Our findings strengthen support for a possible link between melioidosis transmission and water by demonstrating

a strong association between melioidosis case numbers and rainfall amounts 1 week before disease onset and humidity levels 2 weeks before disease onset.

The variations in intervals between rainfall and disease manifestation that we found are within the estimated incubation period of 1–21 days for melioidosis. However, this finding may also be accounted for by the existence of variations in genome sizes, intraspecies diversity, and virulence in the *B. pseudomallei* strains from diverse geographic locations (e.g., Thailand, Vietnam, Singapore, Australia) (13). The association between water and melioidosis is further strengthened by findings from a recent epidemiologic case-control, interview-based survey of patients, which found that exposure to rain and water inhalation were among the risk factors for acquisition of disease (14).

In summary, we found that, in Singapore, a highly urban area where contact with soil is rare, the numbers of melioidosis cases are associated with higher rainfall totals and higher humidity levels in the weeks preceding illness onset. This finding indicates that water, rather than soil, may be the central vehicle for transmission and acquisition of *B. pseudomallei* infection.

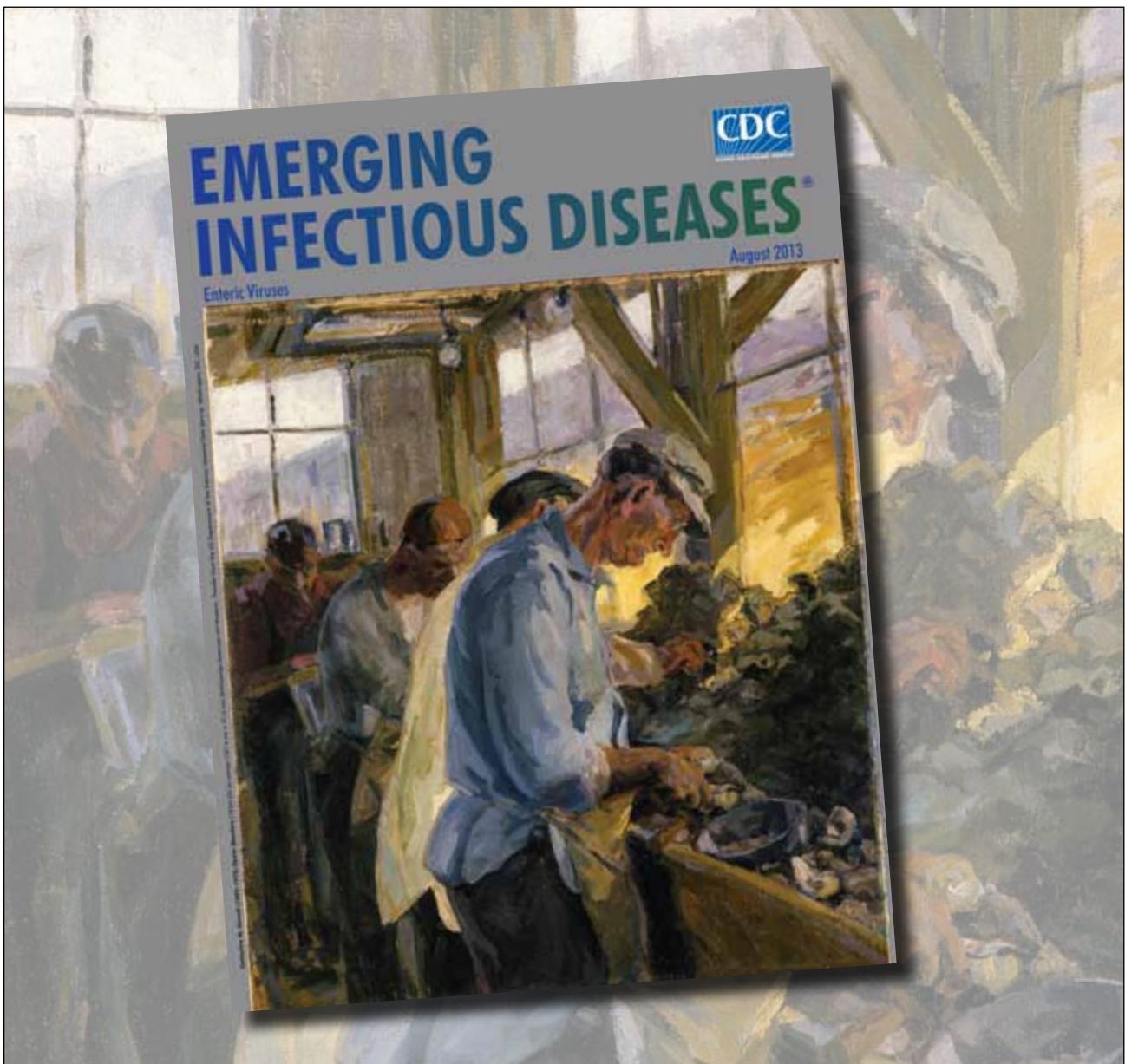
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Noninvasive Detection of *Echinococcus multilocularis* Tapeworm in Urban Area, Estonia

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To the Editor: Alveolar echinococcosis, which is caused by the fox tapeworm *Echinococcus multilocularis*, is an emerging disease in Europe that shows a high mortality rate (1). Humans can become infected after ingesting parasite eggs (e.g., through direct contact with dogs and red foxes [*Vulpes vulpes*] or with their contaminated feces). *E. multilocularis* tapeworm eggs are extremely resistant and can remain viable in the environment for years (2).

Numbers of red foxes have increased in many countries in Europe in recent decades, and the *E. multilocularis* tapeworm has also expanded its range. This tapeworm has recently been reported in 17 countries in Europe, including Lithuania, Latvia, and Estonia (1). Foxes and associated tapeworms are also increasingly found in urban areas, prompting considerable public health concern (1,3). Foxes began to colonize urban areas in Estonia in 2005, and they have since been reported in 33 of 47 towns nationwide (L. Plumer et al. unpub. data). Because ~30% of foxes are

infected with the *E. multilocularis* tapeworm in natural habitats in Estonia (4), it is essential to monitor parasite spillover into urban areas, where it could become a serious public health risk. Consequently, there is an acute need for methods that can effectively detect the parasite and thereby help prevent human infection.

Although immunologic (2) and genetic methods (5–7) are available for identifying *Echinococcus* spp. parasites, a sensitive molecular diagnostic method that detects tapeworms and identifies their host species from degraded fecal samples would be useful. The purposes of this study were to develop a sensitive, noninvasive, genetic method to identify the host species by discriminating between feces of red foxes and dogs; detect *E. multilocularis* tapeworms in feces and distinguish them from the related parasite *E. granulosus*; and collect carnivore feces in an urban area in Estonia to identify this tapeworm.

Fecal samples suspected to be from red foxes were collected during January–March 2012 and January–March 2013 from streets and grassy areas of Tartu, Estonia. Tartu is a relatively small city (area 39 km²) with 98,000 human inhabitants. We surveyed 14 transects, each ~4 km in length, that included all major districts in the city (Figure). Each transect was searched weekly during the study period (total ~850 km surveyed).

A total of 137 fecal samples were collected and stored at –80°C for ≥1 week to avoid risk of infection from any *Echinococcus* spp. eggs present (2) because *E. multilocularis* (4) and *E. granulosus* (8,9) tapeworms have been found in Estonia. Samples of ~250 mg were placed into 2-mL tubes, heated at 65°C for 15 min, and stored at –80°C. The heating and cooling procedure helps to break the parasite egg shells, enabling more efficient DNA

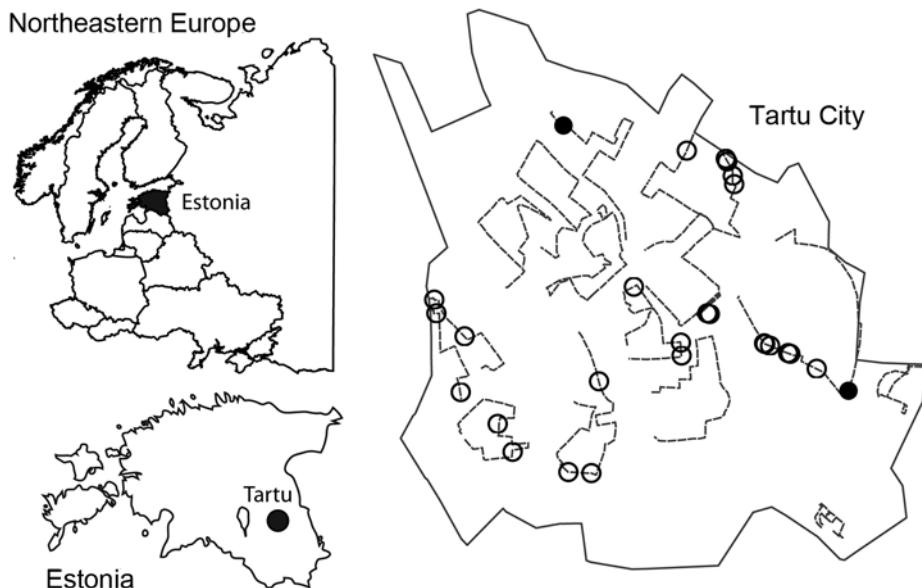


Figure. Location of Tartu in northeastern Europe, Estonia, and red fox feces sampling area in Tartu. The Tartu City boundary is indicated by a solid black line, survey transects are indicated by dashed lines, and fox fecal samples ($n = 28$) are indicated by circles. Filled circles ($n = 2$) indicate samples positive for *Echinococcus multilocularis* tapeworms.

extraction. DNA was extracted by using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions.

Species-specific primers (online Technical Appendix Table, <http://wwwnc.cdc.gov/EID/article/21/1/14-0136-Techapp1.pdf>) were designed to amplify short sequences of mitochondrial DNA. On the basis of primer specificity and amplicon size, we determined host and parasite species (online Technical Appendix Figure 1). DNA extraction and PCR were performed in a laboratory dedicated to environmental samples (for a complete description of methods, see online Technical Appendix).

DNA was successfully extracted and amplified from 119 (86.9%) of 137 fecal samples. Of usable samples, 28 (23.5%) were from red foxes and 91 (76.5%) were from dogs. Two fox fecal samples (7.1%; 95% binomial CIs 0.9%–23.5%) were infected with *E. multilocularis* tapeworms; none of the dog samples were infected.

To verify parasite identification, we amplified DNA from the 2 *E. multilocularis*-positive samples with *E. multilocularis*-specific primers and sequenced the amplification products. To verify host species identification, we used primers that produced longer amplification products (327 and 197 bp) than the corresponding PCR primers and sequenced amplification products from 5 fox samples and 5 dog samples. Sequencing procedures were performed according to the methods of Saarma et al. (10).

Sequences from both *E. multilocularis*-positive samples showed 100% identity with an *E. multilocularis* tapeworm sequence (GenBank accession no. AB018440) (online Technical Appendix Figure 2). All sequenced fox and dog samples also belonged to the corresponding species.

To estimate the sensitivity of this noninvasive genetic method, we determined the number of *E. multilocularis* eggs necessary to obtain a positive PCR result (online Technical Appendix Figure 3). One egg was sufficient to give an *E. multilocularis* tapeworm-specific result.

In summary, we developed a noninvasive genetic method that identifies *E. multilocularis* tapeworms and their host species in carnivore fecal samples found in urban environments. Furthermore, these tapeworms can even be detected in fecal samples from red foxes when only 1 parasite egg is present. Thus, this method is highly sensitive and discriminatory and can be used with degraded fecal samples to monitor *E. multilocularis* tapeworms and their hosts.

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Severe Delayed Hemolysis Associated with Regulated Parenteral Antimalarial Drug

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To the Editor: Intravenous artesunate is recommended by the World Health Organization as first-line treatment for severe malaria. While artemisinin-based therapies are well tolerated, recent studies have reported cases of severe delayed hemolysis after artesunate treatment for malaria (1–5). To date, all reported cases have been associated with the use of artesunate not produced under Good Manufacturing Practice (GMP) standards. The United States and Canada are the only countries that use GMP artesunate, and a recent review concluded that delayed hemolysis may be related to differences in GMP versus non-GMP artemisinins (5). We report a case of severe delayed hemolysis after administration of GMP artesunate to treat a patient with severe malaria.

During October 2012, A previously healthy, 31-year-old Canadian-born man sought treatment at a hospital in Toronto, Ontario, Canada, after 3 days of fever and severe headaches. He had returned 10 days earlier from a 10-day work trip to South Sudan. He did not use malaria chemoprophylaxis while there but did sleep under an insecticide-treated net. During initial assessment, a blood smear showed *Plasmodium falciparum* malaria with parasitemia of 22% (1,100,000 parasites/mL) and the following levels: bilirubin, 88 (reference range 5–17) $\mu\text{mol/L}$; aminotransferase, 105 (reference range 10–38) U/L; creatinine, 130 (reference range 80–115) $\mu\text{mol/L}$; hemoglobin, 144 (reference range 140–160) g/L, and a platelet count of $17 \times 10^9/\text{L}$ (reference range 150–450 $\times 10^9/\text{L}$). In the emergency department, he was given 1 dose each of doxycycline, atovaquone/proguanil, and artemether/lumefantrine; within an hour of ingestion of these drugs, he vomited. He was transferred to a tertiary level hospital for admission to the intensive care unit and exchange transfusion. Intravenous artesunate was administered (2.4 mg/kg at 0, 12, 24, and 48 h), then a 3-day course of oral atovaquone/proguanil was ordered. On admission, his chest radiograph showed no abnormalities, and blood cultures were negative; his hemoglobin level was 125 g/L; no treatment was initiated for decreased hemoglobin. Parasitemia was undetectable within 36 hours of admission to the intensive care unit. The patient was discharged 5 days later.

Four days after discharge, the patient returned to the tertiary level hospital seeking treatment. He reported that beginning 2 days after discharge, he had fever and “merlot-colored” urine. On admission, he was noted to be jaundiced. Laboratory values included levels of bilirubin of 89

$\mu\text{mol/L}$, lactate dehydrogenase (LDH) of 1,976 (reference 120–240) U/L, hemoglobin of 81 g/L and marked hemoglobinuria. Multiple thick and thin blood smears were negative for *Plasmodium* spp.

During the course of his second admission, he required 8 blood transfusions to maintain his hemoglobin level above 75 g/L. He continued to have unexplained hemolysis and hemoglobinuria: laboratory results showed a nadir of hemoglobin at 68 g/L and an LDH peak of 3,429 U/L and a low haptoglobin level (<0.12 g/L [reference 0.3–2.0 g/L]). His glucose-6-phosphate dehydrogenase level was within reference range. Supportive therapy was continued, and hemolysis ceased spontaneously 10 days after onset. When seen during a follow-up visit 6 weeks later, he was asymptomatic and his hemoglobin level was 135 g/L. Pre- and post-transfusion and follow-up testing did not show evidence of red blood cell alloantibodies, making the possibility of a delayed hemolytic transfusion reaction unlikely. Serologic tests showed that he was also positive for causative organisms for schistosomiasis, strongyloidiasis, and Q fever. These diagnoses were consistent with past infections and were not considered to be contributory to the current severe hemolytic event.

In all previous case reports of delayed hemolysis, patients received World Health Organization–prequalified, but not GMP-certified, artesunate (1–5). In this report, the parenteral drug used was GMP certified and produced by the US Army Medical Materiel Development Activity. A diagnosis of artesunate-associated hemolysis was made in this case based on the temporal relationship with therapy and the absence of other identified causes of intravascular hemolysis. His time course of hemolysis after treatment corresponds with recent case series in Europe (1–4): his hemoglobin level reached a nadir at approximately day 15. The outcome of this case corresponds with a proposed case definition by Rolling and colleagues to distinguish artesunate-related hemolysis from that attributable to malaria infection alone (4).

We suggest a case definition whereby a decrease in hemoglobin combined with an increase in LDH between week 2 and 3 is characteristic of delayed hemolysis associated with artesunate. Because treatment for severe malaria is not given as monotherapy, we cannot exclude a potential contributory role of the other antimalarial agents he received. However, severe intravascular hemolysis has rarely been reported in relationship to these agents. Additionally, we cannot exclude a potential role for drug-induced immune hemolysis. Nonetheless, given the severity of the hemolysis and the delayed onset, health care workers should be cognizant of this late, potentially life-threatening complication of artemisinin-based therapy. All patients treated with artesunate for severe malaria should be monitored for 4 weeks and evaluated for hemolytic anemia.

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***Burkholderia pseudomallei* Sequence Type 562 in China and Australia**

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To the Editor: Melioidosis is increasingly being recognized in tropical and subtropical areas worldwide; the world's 2 major endemic foci are Thailand and northern

Australia (1,2). Phylogenetic analyses of *Burkholderia pseudomallei* isolates, performed by using multilocus sequence typing (MLST) (3), have led to phylogeographic associations that can be used to track melioidosis epidemics (4). However, in contrast to the previous separation of *B. pseudomallei* into 2 phylogenetic groups (Australia and Southeast Asia/rest of the world) (5), we report an MLST sequence type (ST) that seems to be present in northern Australia, Taiwan, and southern China.

In mainland China, melioidosis was first reported in 1990 (6) and is now known to be endemic to several tropical provinces, including Hainan, a southern island province close to Southeast Asia. Since 2008, cases of melioidosis in Hainan have escalated; from July 2008 through July 2012, a total of 110 cases were microbiologically diagnosed at 2 general hospitals (Sanya People's Hospital and Haikou Municipal Hospital).

We characterized clinical isolates of *B. pseudomallei* from the 110 cases by using MLST, pulsed-field gel electrophoresis (PFGE), and 4-locus multilocus variable-number tandem-repeat analysis (MLVA-4) (3,7,8). MLST revealed 40 STs, 39 of which were consistent with STs from Southeast Asia, as evident from the global *B. pseudomallei* MLST database (<http://bpseudomallei.mlst.net/>). A single ST, ST562, which accounted for 3 cases in Hainan, was previously described on the global database as being from Australia; the 20 isolates from humans and 10 isolates from the environment deposited until September 1, 2014, all from Australia, had been isolated from 2005 through 2012. Although not deposited in the global MLST database, ST562 has also recently been reported from Taiwan (7). Among the 253 isolates of *B. pseudomallei* collected in Taiwan during 2004–2010, 1 clinical isolate and 9 environmental isolates were described as being ST562. Moreover, these 10 ST562 isolates displayed a unique PFGE pulsotype, distinct from that of other *B. pseudomallei* strains from Taiwan (7).

Of the 3 patients from Hainan from whom ST562 strains were isolated, 2 resided in the city of Sanya and 1 in the neighboring city of Lingshui (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-0156-Techapp1.pdf>); all denied a history of foreign travel, they shared no common risk factors, and all survived the infection. Further analysis of ST562, performed by using eBURST-based (<http://eburst.mlst.net/>) population analysis of the MLST dataset, showed that ST562 is a single-locus variant of ST167, which is represented on the MLST dataset by multiple human and environmental isolates from Thailand and to date by 1 human isolate from Cambodia. ST167 accounted for 1 of the 110 *B. pseudomallei* strains from Hainan. The *narK* locus of ST167 contains allele 3 instead of allele 29, as seen in ST562; 3 base differences are found in allele 3: C72T (C→T position 72), C126T, and A435G. According to PFGE, the 3 ST562

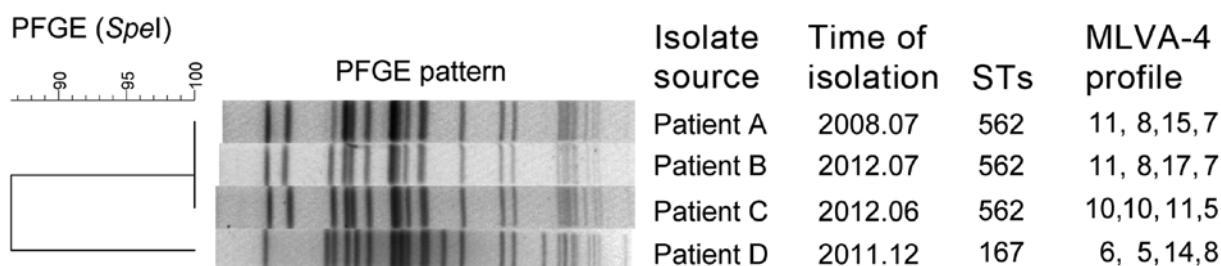


Figure. Pulsed-field gel electrophoresis (PFGE) patterns for 3 sequence type (ST) 562 and 1 ST167 *Burkholderia pseudomallei* strains isolated during 2008–2012, Hainan, China. The isolate source, isolation time, ST, and 4-locus multilocus variable-number tandem-repeat analysis (4-MLVA) profiles are indicated for each strain. Scale bar indicates percentage similarity.

isolates from Hainan displayed a single pulsotype, and the other 107 isolates from Hainan belonged to distinct and diverse pulsotypes, similar to those observed in Taiwan. The uniformity of PFGE patterns in the Hainan and Taiwan isolates supports the possibility that ST562 might be a recently emerging clone. PFGE patterns of Hainan ST562 exhibited 86% similarity with ST167, differing by 6 bands (Figure).

Hainan ST562 isolates were further analyzed by using MLVA-4 (8), which divided 3 isolates (from patients A, B, and C) into 3 distinct MLVA-4 types (Figure). The 2008 isolate (MLVA-4 profile 11,8,15,7) and one 2012 isolate (profile 11,8,17,7) exhibited close relatedness, whereas another 2012 isolate (profile 10,10,11,5) was divergent from these, indicating that ST562 isolates in Hainan have been present long enough for some divergence into lineages.

Two mutually exclusive gene clusters, *B. thailandensis*-like flagellar gene cluster (BTFC) and *Yersinia*-like fimbrial gene cluster (YLF), have been linked to geographic origin and have been suggested for differentiating groups of *B. pseudomallei* (9). By PCR we found that ST562 isolates of Hainan were all YLF positive. BTFC predominates in Australian *B. pseudomallei* strains, and YLF predominates in Southeast Asia. Presence of YLF was also observed in strains from Papua New Guinea, possibly reflecting that country's location, intermediate between major foci of melioidosis (10).

In conclusion, by using MLST and the online MLST database, we revealed that *B. pseudomallei* ST562 is present in southern China as well as in Australia and Taiwan. The intercontinental character of this ST raises new questions about the epidemiology and control of melioidosis. Given the usual geographic separation of *B. pseudomallei* STs, we suggest that this wide-ranging presence of ST562 might result from more recent spread caused by transmission between regions. Increasing farming exchanges and trade of agricultural products between melioidosis-endemic regions might facilitate breaking of the geographic barrier;

clonal introduction of *B. pseudomallei* could potentially occur in new locations. Improved and cooperative surveillance is required for elucidating the current and future global dispersion range of *B. pseudomallei* and for monitoring the consequent melioidosis infections.

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Hemolytic Uremic Syndrome Associated with *Escherichia coli* O8:H19 and Shiga Toxin 2f Gene

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To the Editor: Gastroenteritis caused by Shiga toxin-producing *Escherichia coli* (STEC), associated with hemorrhagic colitis and hemolytic uremic syndrome (HUS), has been identified as a major health problem (1). Shiga toxin is essential for the development of HUS (2). Shiga toxin can be distinguished into Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). The *stx*_{2f} STEC variant is a distinct group within STEC (regarding virulence genes) and is known to cause relatively mild disease, although reports of human illness are scarce (3).

During autumn 2013, a healthy 9-year-old boy in the Netherlands experienced fever, vomiting, and bloody diarrhea which persisted for days; he was admitted to the pediatric ward of a local hospital because of clinical signs of HUS with renal insufficiency: serum creatinine level 439 μmol/L (reference range 31–68 μmol/L); blood urea nitrogen concentration 34.1 mmol/L (reference range 3.3–5.6 mmol/L); thrombocytopenia (46 platelets/nL); reference

range 150–450/nL), and low haptoglobin level. Hemoglobin levels decreased within 48 hours from 7.4 mmol/L to 5.5 mmol/L (reference range 6.9–8.4 mmol/L). His blood pressure was 127/82 (99th percentile for age and height). Renal insufficiency worsened over time, evidenced by maximum urea levels of 57.3 mmol/L and maximum creatinine levels of 744 μmol/L. Vomiting increased, and feeding became difficult. The boy was transferred to an academic nephrology center, where he received erythrocyte and thrombocyte infusions, then peritoneal dialysis. He received 1 prophylactic dose of cefazolin during insertion of the dialysis catheter. After 2 days, he entered a polyuric phase of renal failure; renal function normalized within a few weeks, however. To improve proteinuria, physicians prescribed a 3-month course of angiotensin-converting enzyme inhibitors after discharge.

A fecal sample tested positive for STEC by PCR in a local laboratory. Five isolates were sent to the National Institute for Public Health and the Environment (RIVM) as part of the national STEC surveillance. By using PCR, 1 of the 5 tested positive for the *stx*_{2f} gene and the attaching and effacing gene (*eae*), and negative for the genes *stx*₁, *stx*_{2a-e}, H7, O157, and enterohemorrhagic *E. coli* hemolysin (*hly*). Serotyping identified O8:H19. The other 4 isolates tested negative for all of the above-mentioned genes and were not serotyped.

The family had stayed in a hotel in Turkey and returned to the Netherlands 5 days before onset of illness. The only reported contact with animals was with a parrot in the hotel. On return to the Netherlands, the boy had eaten filet américain, a sandwich spread made of raw beef. The day before disease onset, he attended a party where barbecue was served by a catering company.

Since 2007, besides this reported case, 8 cases of STEC O8 were registered within the STEC surveillance system in the Netherlands: O8:H– (4 cases), O8:H19 (2 cases), O8:H8 (1 case), and O8:H9 (1 case). All 8 isolates were *stx*_{2a-e}-positive and *stx*₁-, *stx*_{2f}-, *eae*-, and *hly*-negative. Disease associated with these cases was relatively mild. During 2007–2010, a total of 13,545 human STEC infections were reported in Europe: 20 were registered as STEC O8; HUS did not develop in these case-patients (4). HUS developed in 2 patients infected with STEC O8 (O8:H2, O8:H19) in Germany during 1996–2000 (5); these isolates and all other isolates from HUS and non-HUS case-patients in this period tested negative for *stx*_{2f}. During 2008–2011, 87 *stx*_{2f} STEC infections were registered in the Netherlands (3). These infections were relatively mild; no HUS cases were registered. The virulence genes seen in the isolate of the described case, *stx*_{2f} and *eae*, but no *hly* or other toxin genes, were also seen in 97% of *stx*_{2f} STEC infections reported in the Netherlands (3). Besides being detected in humans, *stx*_{2f} STEC has only been detected in pigeons (6).

The cause of the severity of disease in this *stx_{2f}* STEC case and the source of the infection could not be determined. The parrot in the hotel in Turkey could have been the source if birds are a reservoir of *stx_{2f}* STEC. Conversely, the uncooked beef and barbecue cannot be ruled out, because O8:H19 has been found in cattle, pigs, and sheep (7). This case shows that STEC subgroups known to cause relatively mild disease can occasionally cause severe disease and that surveillance based upon a small group of serotypes underestimates the number of severe STEC infections and increases the chance of missing emerging serotypes.

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Monitoring Water Sources for Environmental Reservoirs of Toxigenic *Vibrio cholerae* O1, Haiti

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To the Editor: In the March 2014 issue of *Emerging Infectious Diseases*, Alam et al. reported a survey of water sources in Haiti conducted to isolate *Vibrio cholerae* (1). Each month from April 2012 through March 2013, they sampled 15 sites at 3 rivers and 1 estuary in West Department. From 179 water samples and 144 aquatic animals and plants, they obtained 7 *V. cholerae* O1 isolates, including 3 *ctx*-positive toxigenic strains.

Unfortunately, the results for all 7 *V. cholerae* O1 isolates were aggregated, and no details were provided about the exact time and location of collection of samples corresponding to the 3 *ctx*-positive strains. The authors posed the question of whether *V. cholerae* O1 has become established in environmental reservoirs in Haiti, subsequently warning that “as long as the causative microorganism is present in the environment, eradication of the disease will not be possible.”

However, after challenging their results with more accurate epidemiologic data, we found that these 3 *ctx*-positive toxigenic strains could more likely have been present in the sampled rivers as a result of recent fecal contamination (Figure, <http://wwwnc.cdc.gov/EID/article/21/1/14-0627-F1.htm>). Indeed, many cholera cases were reported in the corresponding communal sections (i.e., the smallest Haitian administrative unit, average 25 km²) when the samples containing the 7 *V. cholerae* O1 isolates were collected. In this context of an ongoing cholera epidemic associated with persisting rainfall (Figure), generalized open-air defecation inevitably leads to contamination of water sources. It is therefore impossible to determine whether *V. cholerae*-positive rivers constitute perennial reservoirs of the bacteria or whether they act only as transient vectors of the pathogens.

The recent dramatic decrease in cholera transmission may provide a good opportunity to address this issue (2). We thus encourage Alam et al. to continue the search for *ctx*-positive toxigenic *V. cholerae* O1 strains in surface waters, especially during cholera-free periods.

Acknowledgments

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Reservoir Host Expansion of Hantavirus, China

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To the Editor: Hemorrhagic fever with renal syndrome (HFRS) is caused by hantavirus. During 1995–2005, China reported 20,000–50,000 cases of HFRS annually, which represents 90% of HFRS cases worldwide (1–3). In China, HFRS is caused mainly by 2 serotypes of hantavirus: Hantaan virus (HTNV) and Seoul virus (SEOV) (4). Pathogenic hantavirus serotypes are considered to be strictly associated with their serotype-specific reservoir hosts. HTNV is associated with the striped field mouse (*Apodemus agrarius*), and SEOV is associated with the brown rat (*Rattus norvegicus*) and the black rat (*Rattus rattus*) (4,5). HTNV causes a severe form of HFRS, characterized by renal failure that in some cases is followed by pulmonary edema and disseminated intravascular coagulation; the estimated death rate is 5%–15%. SEOV causes a moderate form of HFRS (6).

Jiaonan County in Shandong Province is one of the high-incidence HFRS areas in China. To detect the hantavirus infection in small mammals, we trapped rodents and shrews during December 2012–November 2013 using snap-traps in Jiaonan County (longitude 119°30′–120°30′, latitude 35°35′–36°08′).

We captured 1,276 animals comprising 5 rodent species and 1 shrew species (Table) and analyzed serum antibody against hantavirus of each animal using an antigen sandwich

ELISA Kit (Shanghai Jiahe Biotechnology, Shanghai, China). The serum was considered to contain antibodies against hantavirus when the optical density (OD)_{450nm} of the sample was greater than the threshold. The threshold was calculated by using the equation: threshold = the average OD of the negative control + 0.15. ELISA results showed that 23.3% of animals were seropositive to hantavirus antigen (Table). The seropositive rate to hantavirus was 44.0% in Asian house shrews (*Suncus murinus*), 25.3% in house mice (*Mus musculus*), 15.4% in Chinese hamsters (*Crictulus griseus*), 10.3% in brown rats, 10.1% in striped field mice (*Apodemus agrarius*), and 3.0% in greater long-tailed hamsters (*C. triton*). The seropositivity rate for rodents was higher during summer (May–August) and lower during spring (March and April) and winter (October and November) but not significantly different among the months.

To determine what types of hantavirus infected the animals, we amplified viral RNA of HTNV and SEOV from animal lung samples using reverse transcription PCR with serotype-specific primers (7); 2.1% of animals had viral RNA of HTNV, and 2.1% had viral RNA of SEOV (Table). HTNV RNA was detected in striped field mice (6.3%), house mice (1.4%), and brown rats (0.6%). The hantavirus-positive animals were captured in February, April, and November for striped field mice; November for brown rats; and April and November for house mice. SEOV was detected in brown rats (8.2%) and Asian house shrews (1.7%). These SEOV-positive animals were captured in January, March, May, June, and July for brown rats and March and November for Asian house shrews. The phylogenetic analysis of sequences amplified by reverse transcription PCR is presented in the online Technical Appendix Figure (<http://wwwnc.cdc.gov/EID/article/21/1/14-0960-Techapp1.pdf>). The nucleotide sequences of the PCR products have been deposited in GenBank (accession nos. KM357423–KM357452).

Hantavirus had been considered to be strictly associated with specific reservoir hosts and to have the same geographic distribution pattern as these reservoir hosts. All hantaviruses that caused human diseases had been associated with rodents, including members of *Murinae*, *Arvicolinae*, and *Sigmodontinae* spp. Insectivore hantaviruses were not known to cause human disease. The rodent hantavirus and the insectivorous hantaviruses were thought to have co-evolved with their specific rodent and insectivorous hosts over millions of years (8). One observed geographic clustering of hantavirus strains, and the association of hantaviruses with their reservoirs, might have been caused by an isolation-by-distance mechanism (9,10) and mixture of both host switching and co-divergence (10). Our study demonstrated that HTNV not only infects its traditional host, the striped mouse, but also infects house mice and rats; SEOV infects not only rats but also shrews, suggesting host expansion for both HTNV

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Table. Seropositive rate and RT-PCR–positive rate of hantaviruses in small mammals, Jiaonan County, China, December 2012–November 2013*

Animal species	No. (%) animals	Seroprevalence of hantavirus	No. tested/no. RT-PCR positive (%)	
			HTNV	SEOV
<i>Apodemus agrarius</i>	268 (21.0)	27 (10.1)	12/191 (6.3)	0/191
<i>Cricetulus griseus</i>	156 (12.2)	24 (15.4)	0/63	0/63
<i>C. triton</i>	135 (10.6)	4 (3.0)	0/48	0/48
<i>Mus musculus</i>	245 (19.2)	62 (25.3)	2/143 (1.4)	0/143
<i>Rattus norvegicus</i>	213 (16.7)	22 (10.3)	1/159 (0.6)	13/159 (8.2)
<i>Suncus murinus</i>	259 (20.3)	114 (44.0)	0/121	2/121 (1.7)
Total	1,276 (100)	253 (19.8)	15/725 (2.1)	15/725 (2.1)

*HTNV, Hantaan virus; RT-PCR, reverse transcription PCR; SEOV, Seoul virus.

and SEOV in China. Our hypothesis is that the hantaviruses co-evolved with their animal hosts, such as SEOV with rats and HTNV with striped mice, but when their animal hosts expanded their territory, hantavirus had more chance to infect other susceptible rodents and expanded their animal hosts.

Both Asian house shrews and house mice are closely associated with humans by living inside and outside of human houses in China. The Asian house shrew and house mouse have been underestimated as potential animal hosts of SEOV and HTNV. To our knowledge, only 1 previous study had associated Asian house shrews with SEOV; in that study, an SEOV strain was isolated from an Asian house shrew in China (2).

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Endophthalmitis Outbreak Associated with Repackaged Bevacizumab

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To the Editor: An outbreak of endophthalmitis associated with repackaged bevacizumab occurred during February–March 2013 in Georgia and Indiana, USA. Bevacizumab (Avastin; Genentech, Inc., South San Francisco, CA, USA) is a vascular endothelial growth factor inhibitor that is approved by the US Food and Drug Administration as an antineoplastic agent but is commonly used off-label to treat retinal disorders, including age-related macular degeneration (1,2). Bevacizumab is manufactured in single-use,

preservative-free, 4-mL vials; compounding pharmacies repackage bevacizumab into syringes for intraocular administration at smaller doses (e.g., 1.25 mg bevacizumab in 0.05-mL injection). Repackaged bevacizumab has been linked to endophthalmitis outbreaks worldwide in which compounding procedure deficiencies have led to microbial contamination and subsequent endophthalmitis (3–7). Endophthalmitis often results in vision loss, particularly if the infection is not identified early and treated aggressively (4–6).

During March 6–8, 2013, four patients with age-related macular degeneration received a diagnosis of acute endophthalmitis after receiving intravitreal bevacizumab injections on March 4, 2013, at a retinal specialty clinic (clinic A) in Georgia. All 4 patients were injected with bevacizumab from the same lot (lot Z), which was repackaged at a Georgia compounding pharmacy (pharmacy A) on February 13, 2013. The Georgia Department of Public Health (DPH) and the Georgia Drug and Narcotics Agency (GDNA) were notified of the outbreak by clinic A, and the outbreak was investigated to determine the extent and source of infections and to prevent additional cases.

Cases were defined as acute endophthalmitis occurring among patients ≤ 14 days after they received intraocular injection with bevacizumab that had been repackaged at pharmacy A after December 18, 2012. Pharmacy A records indicated that bevacizumab had been distributed to 11 clinics in 4 states; state public health authorities were notified, and facilities that received the medication were contacted and asked to report cases to DPH. Clinic A identified 60 additional patients who received bevacizumab from lot Z and monitored them for signs of infection. One additional patient with endophthalmitis was identified in Indiana; this patient was injected on February 22 with bevacizumab that had been repackaged at pharmacy A on February 13, 2013.

DPH epidemiologists evaluated clinic A infection prevention practices, including use of face masks and sterile techniques during injection procedures; examined possible sources of contamination; and reviewed case-patient medical records. DPH and GDNA evaluated pharmacy A, including its equipment and sterile compounding procedures. Vitreal fluid samples were collected from all case-patients and were cultured on chocolate agar medium.

The median age of the 5 case-patients was 80 years (range 59–89 years); 3 case-patients were men. Postprocedural signs and symptoms included pain, vision loss, retinal hemorrhage, hypopyon, and vitreous haze. Patients who received a diagnosis of endophthalmitis were treated with pars plana vitrectomy or vitreal tap; intraocular injection of vancomycin with gentamicin, ceftazidime, or ceftazidime and amphotericin; and oral moxifloxacin. All patients regained vision in the affected eye.

Evaluation of clinic A found appropriate mask and glove use and no deficiencies in bevacizumab injection

technique or medication storage and handling. Investigation of pharmacy A revealed multiple areas in which practices did not conform to United States Pharmacopeial Convention Chapter 797 standards for compounding sterile preparations or to recommended best practices for repackaging bevacizumab; these deficiencies might have contributed to bevacizumab contamination (6,8). GDNA suspended pharmacy A from performing sterile compounding until compliance with these standards.

Culture of vitreous fluid samples from all patients in Georgia grew *Granulicatella adiacens*, a gram-positive bacterium that is part of the oral flora but a rare human pathogen not previously reported to cause endophthalmitis (9). The Indiana patient was infected with *Abiotrophia* (not further speciated); *Granulicatella* and *Abiotrophia* are similar bacterial genera that are difficult to distinguish by morphologic features (10).

Contamination introduced during repackaging at pharmacy A was the likely source of this outbreak. This conclusion was supported by evidence of common or similar organisms among 5 patients from 2 states after injection with bevacizumab repackaged on the same date at pharmacy A, combined with documented deficiencies in pharmacy A's sterile compounding processes.

Acute postinjection endophthalmitis can occur when intraocular bevacizumab is used because of risks associated with repackaging contents from single-use, preservative-free vials. Ensuring adherence to standards for sterile compounding is critical for preventing contamination and providing patients with a safe source of intraocular bevacizumab (6). If this drug were available from the manufacturer in appropriately sized, prefilled syringes or containers, risks associated with repackaging and mishandling might be eliminated (2,6). Ophthalmology clinics that rely on repackaged bevacizumab for intraocular use should be vigilant in selecting pharmacies to perform this service. Resources are available to assist facilities in assessing the quality of outsourced sterile compounding services (e.g., <http://www.ashpfoundation.org/MainMenuCategories/PracticeTools/SterileProductsTool/SterileProductsAssessmentTool.aspx>). In addition, ophthalmologists should adhere to aseptic techniques and safe injection practices (http://www.cdc.gov/injectionsafety/ip07_standardprecaution.html) when preparing or administering intraocular injections. A single case of endophthalmitis may signal a more widespread problem; prompt reporting to public health authorities, investigation, clinician engagement, and product recalls can be critical for limiting patient harm.

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Diverse *Francisella tularensis* Strains and Oropharyngeal Tularemia, Turkey

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To the Editor: Tularemia is a zoonosis caused by the bacterium *Francisella tularensis*; the main forms of disease that occur in humans are ulceroglandular/glandular, oculoglandular, oropharyngeal, and respiratory. In Turkey, tularemia outbreaks were described as early as 1936–1938 (1), but tularemia was not reportable until 2004. Recently, multiple tularemia outbreaks in Turkey have been described, including in regions where the disease has not been previously reported; it is now considered a reemerging zoonotic disease in Turkey (1).

The only *F. tularensis* subspecies found in most of Eurasia, including Turkey, is *holarctica*. Genetic diversity is low, probably because emergence is recent (2). However, discovery of whole-genome single-nucleotide polymorphisms (SNPs), coupled with subsequent canonical SNP (canSNP) analyses, have identified numerous phylogenetic groups within this subspecies. The distinct phylogeographic patterns provide insight into its evolutionary history (3–7).

From December 2009 through January 2011, tularemia outbreaks increased in Turkey, primarily in the central region (8). Oropharyngeal tularemia was diagnosed for

Table. *Francisella tularensis*–positive clinical samples from 14 patients with oropharyngeal tularemia, Turkey, December 2009–January 2011*

Patient no. (sample no.)	City	CanSNP subgroup†	MLVA genotype‡
1 (F0737)	Corum	B.20/21/33	i
2 (F0738)	Cankır	B.28/29	b
3 (F0739)	Yozgat	B.28/29	b
4 (F0740)	Zonguldak	B.7/8	a
5 (F0741)	Corum	B.20/21/33	e
6 (F0742)	Corum	B.20/21/33	e
7 (F0743)	Corum	B.20/21/33§	ND
8 (F0744)¶	Bala/Ankara	B.20/21/33	e
9 (F0745)	Ankara	B.20/21/33	d
10 (F0746)	Corum	B.20/21/33	j
11 (F0747)	Bala/Ankara	B.20/21/33	g
12 (F0748)	Corum	B.20/21/33	f
13 (F0749)	Ankara	B.20/21/33	c
14 (F0750)	Emirdağ/Afyon	B.20/21/33	h

*Clinical samples collected in 2011 from patients with cervical lymphadenitis at Hacettepe University Medical Faculty, Pediatric Infectious Disease unit, Ankara, Turkey. CanSNP, canonical single-nucleotide polymorphism; MLVA, multilocus variable-number tandem-repeat analysis; ND, not determined.

†Subgroups published in (3,4,6,7). CanSNP branches tested on samples in this study: B.3, B.4, B.5, B.6, B.7, B.8, B.9, B.10, B.11, B.13, B.20, B.21, B.22, B.26, B.27, B.28, B.29, B.30, B.31, B.32, and B.33.

‡MLVA markers (M03, M05, M06, M20) (2).

§Subgroup classification based on approximation because genotype for B.33 remains unresolved.

¶This patient was an adult (all others were children).

¹These authors contributed equally to this article.

14 patients (13 children, 1 adult), and fine-needle lymph node aspiration was performed at the Pediatric Infectious Diseases Unit at Hacettepe University, Ankara. DNA was extracted from these 14 samples (QIAamp DNA Mini Kit; QIAGEN, Hilden, Germany) and screened by using a PCR selective for the *tul4* gene region specific to *F. tularensis* (9); all 14 samples were positive for *F. tularensis* (Table). Residual, de-identified portions of these 14 DNA extracts were used for this study.

Genetic characterization led to assignment of these 14 samples to multiple phylogenetic groups within *F. tularensis* subsp. *holarctica*. Analysis with 18 previously described (3,4,6,7) canSNP assays (Table) led to assignment of the 14 samples to 3 previously described phylogenetic groups within this subspecies: B.20/21/33 (n = 11), B.28/29 (n = 2), and B.7/8 (n = 1) (Table; online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/1/14-1087-Techapp1.pdf>). To identify additional genetic diversity, we used 4 previously described (2) variable-number tandem-repeat markers (M03, M05, M06, and M20) and identified 10 genotypes among the 14 samples, 8 of which were identified in the 11 B.20/21/33 samples (Table).

The genetic diversity among these samples and their widespread geographic origins from 6 provinces in central Turkey (Table; online Technical Appendix) suggest that the patients contracted tularemia from multiple independent sources. These sources might have been contaminated drinking water, which has been implicated as the source of human tularemia in previous outbreaks in Turkey (1) and could account for oropharyngeal tularemia in the 14 patients reported here.

The finding of these 3 phylogenetic groups within Turkey expands the known geographic range of these phylogenetic groups within *F. tularensis* subsp. *holarctica*. The presence of group B.28/29 *F. tularensis* in Turkey is not surprising; isolates belonging to this group were previously identified in bordering Georgia (3). Likewise, the presence of group B.20/21/33 *F. tularensis* is not unexpected, given the wide geographic distribution (Sweden, Finland, Russia, and Hungary [4]) of organisms belonging to this group (online Technical Appendix). Isolating group B.7/8 *F. tularensis*, previously thought to occur only in Scandinavia, in Turkey is of particular interest, given the relatively basal position of this group in the *F. tularensis* subsp. *holarctica* phylogeny (online Technical Appendix). Indeed, descendants of this group have, to date, been identified from North America only, suggesting a transfer from the Old World to the New World within this lineage (7). The circumstances of this transfer are unknown but might be discerned through additional knowledge of the geographic extent and genetic diversity of organisms in the B.7/8 group.

It has been suggested that Scandinavia might be the source of the historical spread of tularemia to the rest of

Europe and might be the origin of the ancestor to the B.13 clade (5). This suggestion was previously argued because *F. tularensis* subsp. *holarctica* isolates from Sweden have yielded more phylogenetic diversity than isolates from any other country. Indeed, except for the B.27 clade, much of the known phylogenetic diversity of this organism within Europe is present in Sweden (5). Some of the largest sets of analyzed samples originated in Sweden (5–7), whereas eastern Europe and much of Asia remain mostly undersampled. The high genetic diversity identified in our very limited sample set from Turkey is notable and includes 2 major lineages (B.7 and B.13; online Technical Appendix). These findings, together with the recent discovery that organisms of multiple *F. tularensis* subsp. *holarctica* phylogenetic groups exist in China (10), suggest that much additional phylogenetic diversity within this subspecies remains to be discovered in Eurasia, which will provide better information about the evolutionary history and historical spread of *F. tularensis* subsp. *holarctica*.

We have demonstrated that high-resolution genetic characterization of *F. tularensis* DNA extracted from biopsy samples is possible, and we conclude that oropharyngeal human tularemia in Turkey is caused by organisms of multiple distinct phylogenetic groups within this subspecies. This pattern, together with the wide geographic distribution of the 14 patients within Turkey (online Technical Appendix), suggests that the persons infected by *F. tularensis* during the 2009–2011 outbreaks in Turkey obtained their infections from multiple environmental sources.

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Dengue Virus Serotype 3 Infection in Traveler Returning from West Africa to Germany

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To the Editor: Dengue virus (DENV) is a member of the family *Flaviviridae*, genus *Flavivirus*, and comprises 4 serotypes (DENV-1, DENV-2, DENV-3, and DENV-4). DENV is transmitted by *Aedes* spp. mosquitoes in subtropical and tropical countries; an estimated 390 million dengue infections occurred worldwide in 2010 (1). In Africa, locally acquired dengue cases have been reported from 22 countries during 1960–2010, and there is evidence of transmission in 34 countries (2). The burden of dengue in Africa was recently estimated to be in the same range as that in Latin America (1).

In 2013, a 71-year-old man came to the Tropical Medicine Clinic at Heidelberg University Hospital (Heidelberg,

Germany) with suspected dengue fever 3 days after returning from West Africa to Germany. The patient had traveled for ≈6 weeks from mid-September through mid-October 2013 to Togo (Lomé, first 3 weeks), Benin (Ouidah, 1 week), back to Togo (Lomé, 1 week), and Burkina Faso (Ouagadougou, 3 days).

Tests results for DENV nonstructural protein 1 and IgM against DENV were positive. The result of a real-time reverse transcription PCR for DENV 1–4 was also positive. A serotype-specific real-time reverse transcription PCR identified DENV-3 (3,4).

To obtain the sequence of a 1,479-nt fragment of the complete gene of the envelope glycoprotein gene, we designed generic primers specific for all complete DENV-3 genomes available from GenBank (alignment was performed by using Geneious version 6.1; <http://www.geneious.com>), which were then sequentially adapted to sequences obtained (primers and protocol available upon request). Sequencing of the complete envelope glycoprotein gene of the virus isolated from the patient identified DENV-3 genotype III (GenBank accession no. KJ922394).

For phylogenetic comparison, we chose all DENV-3 sequences available from Africa and neighboring regions and a set of global sequences that represented different genotypes; DENV-1 was used as an outgroup. Sequences were aligned on the basis of translated nucleotide sequences, and a neighbor-joining tree with p-distance was inferred with 1,000 bootstrap replicates in MEGA version 5.2.1 (<http://www.megasoftware.net/>) for a 1,479-nt fragment spanning the complete gene of the envelope glycoprotein and a smaller fragment of 220 nt (Figure). We observed clustering of the virus sequence with those of strains from Côte d'Ivoire and Benin. Genetic identity was 99% with strains from Côte d'Ivoire (AB447989) (1,472/1,479 nt) and Benin (AB690858) (1,469/1,479nt).

Because of limited availability of only 4 complete envelope glycoprotein gene sequences from Africa, an additional phylogenetic analysis was performed with a smaller fragment of 220 nt to include more sequences of African origin. A total of 7 sequences, including additional sequences from Senegal and Cameroon, were available. Clustering of sequences of African origin was confirmed in this analysis; highest sequence identity of virus isolated from the patient was with viruses isolated in Côte d'Ivoire, Benin, and Senegal.

Nearly all sequence data for DENV-3 from Africa originate from returning travelers, such as reports of imported cases in 2006 from Cameroon to Spain (5), from Senegal to Spain in 2007 and to Italy in 2010 (5,6), from Côte d'Ivoire to France and Japan in 2008 (7,8), from Benin to Japan and France in 2010 (8,9), and from Eritrea to Finland in 2010 (5). Phylogenetic analysis of virus strains

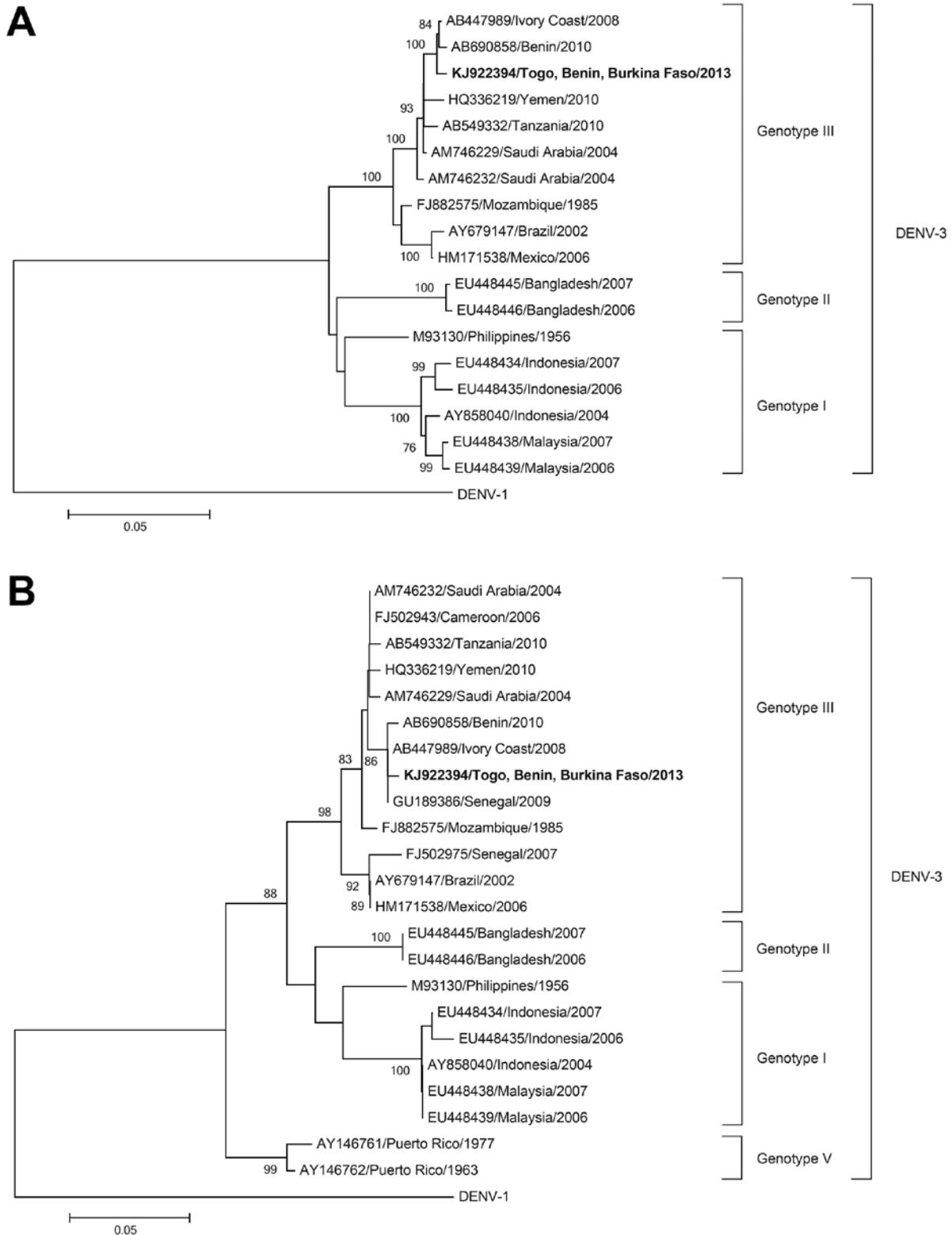


Figure. Phylogenetic trees of A) a 1,479-nt fragment of the complete envelope glycoprotein gene and B) a 220-nt fragment of the partial envelope glycoprotein gene of dengue virus. Phylogeny was based on a neighbor-joining tree with p-distance and 1,000 bootstrap replicates. Bootstrap values >75 are indicated along the branches. The strain isolated in this study from a 71-year-old man who returned from West Africa to Germany is indicated in bold. Scale bars indicate percentage of nucleotide distance.

identified in these cases shows clustering with sequences of African origin, as observed for our patient (6–8). However, because only a small number of sequences of DENV-3 strains from Africa are available (and not all sequences refer to the same genomic region), a comparative phylogenetic analysis of strains from Africa is limited. A recent investigation of febrile patients from Gabon showed not only circulation of DENV-3, but simultaneous circulation of 3 DENV serotypes (DENV-1, DENV-2, and DENV-3) in West Africa (10).

Molecular data for travelers are useful in areas where DENV diagnosis and surveillance are not routinely performed. The case-patient reported here highlights sustained transmission of DENV-3 genotype III strains or closely related strains during recent years. Increasing numbers of reports on local outbreaks and available phylogenetic information support ongoing DENV-3 transmission in West Africa. If one assumes a maximum incubation time of 14 days, our case-patient was most probably exposed to DENV in Togo or Burkina Faso. These 2 countries have not been considered as areas to which DENV is endemic. Our findings indicate that further systematic evaluation of the risk and disease burden of dengue in Africa is urgently needed. Dengue fever should be considered in travelers returning from Africa with acute febrile illness.

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Community-Acquired Invasive GAS Disease among Native Americans, Arizona, USA, Winter 2013

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To the Editor: Group A streptococci (GAS) can cause severe invasive diseases, such as necrotizing fasciitis, streptococcal toxic shock syndrome, and sepsis. In 2012, ≈11,000 cases of invasive GAS (iGAS) disease and 1,100 associated deaths occurred in the United States (1,2). The risk for iGAS infection is 10 times higher among Native Americans than among the general population (3). Other predisposing factors for iGAS infection include skin wounds and underlying diseases, such as diabetes (1,3,4). Household risk factors include exposure to children with pharyngitis and crowding (4). Most iGAS infections occur sporadically within the community. Postpartum and postsurgical clusters arising from a common nosocomial source occur but are rare (5).

During the winter of 2012–13, a 3-fold increase in necrotizing fasciitis was observed at an Arizona hospital

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(hospital X) that predominantly treats Native Americans. Tribal leadership initiated a collaborative investigation with state and federal officials to characterize the outbreak and implement appropriate control measures.

A confirmed case of iGAS was defined as isolation of GAS from normally sterile sites (i.e., blood) or isolation of GAS from nonsterile sites (i.e., wound) in the presence of necrotizing fasciitis or streptococcal toxic shock syndrome among patients who sought care at hospital X during August 2012–March 2013. Hospital X serves ≈45,000 persons in a rural community. Eleven confirmed iGAS cases were identified (Figure), of which 8 (73%) occurred in women and 3 (27%) occurred in men. The case-patients had a mean age of 63 years (range 32–92 years). All cases were community-onset illnesses; none of the case-patients had recent exposures to health care settings, and all were of Native American ancestry. Of the 11 case-patients, 8 required critical care treatment and 3 died. Nine (82%) case-patients had open wounds or skin breakdown (e.g., skin abrasion, burns), and 9 had underlying medical conditions that are known risk factors for iGAS (e.g., obesity, diabetes, chronic kidney or heart disease, alcoholism).

Five GAS isolates were available. Two of the isolates were *emm* type 11; antimicrobial drug–susceptibility profiles for the 2 were identical (i.e., tetracycline resistant). The 2 patients reported no close contact with each other, but they had the same home health aide. The other 3 isolates had different *emm* types (1, 12, and 82) and were antimicrobial drug pansensitive.

We interviewed 58 household contacts of the case-patients (35 adults, 23 children) regarding symptoms and risks for secondary GAS infection. Among these contacts, 2 adults reported a sore throat and 6 children reported

fever (without sore throat), but no confirmed secondary GAS infections were identified. Because of the known increased risk for iGAS among Native Americans and the level of crowding (average of 2–3 persons/bedroom) and the high proportion of adult household contacts with predisposing underlying conditions (29%) in this population, azithromycin prophylaxis was offered to household contacts who spent ≥24 hours with a case-patient during the 7 days preceding the onset of illness.

With the exception of the 2 case-patients with a common health aide, we found no common epidemiologic links or common behaviors among patients that suggested a single-source outbreak. This was further supported by the finding of multiple *emm* types among the isolates. These are not unusual findings in community outbreaks of iGAS; clusters of iGAS cases have often been observed without a common source (6–8). Localized and transient increases in sporadic GAS infections may occur because of an influx of a new *emm* type into a population with low levels of community immunity to that specific *emm* type; an increase in the detection and reporting of iGAS without a true increase in infection; or an increase in conditions that predispose persons to iGAS, such as GAS pharyngitis among children or concurrent influenza or other virus outbreaks in the community.

Past studies have shown that the risk of secondary iGAS infection among household contacts of patients with iGAS disease is higher than that among the general population but still low (5). Although Centers for Disease Control and Prevention guidelines do not recommend routine chemoprophylaxis for household contacts of patients with iGAS infection, the guidelines state that providers may choose to offer antimicrobial drug prophylaxis

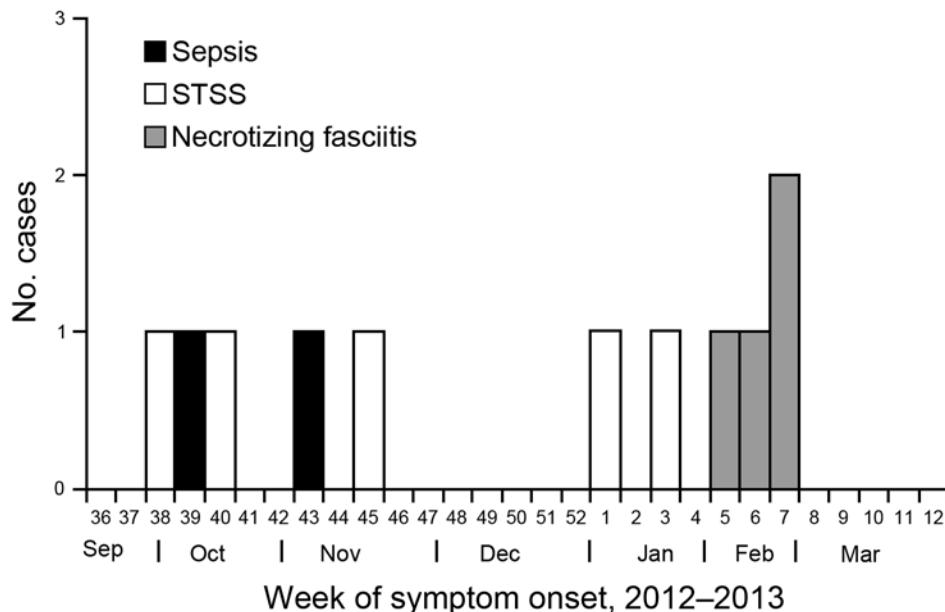


Figure. Week of symptom onset and principal clinical syndrome of patients with confirmed invasive group A streptococcus infections at hospital X, Arizona, August 2012–March 2013. STSS, streptococcal toxic shock syndrome.

to those household contacts at increased risk for iGAS infection (5). Because Native Americans have increased rates of iGAS disease, compared with those of the general population, and because households in this investigation were crowded and many contacts had predisposing underlying conditions, we recommended that household contacts receive prophylaxis if given within 30 days of the index case-patient's illness (5). No additional cases were reported at least 3 months after the investigation and intervention.

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Multidrug-Resistant Tuberculosis Outbreak in Gaming Centers, Singapore, 2012

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To the Editor: Local area network (LAN) gaming centers (variant of cyber cafes) have proliferated over the past 2 decades. Patrons sometimes spend considerable time playing multiplayer computer games at these centers. We report a 2012 outbreak of multidrug-resistant tuberculosis (MDR TB) in Singapore, in which transmission occurred among 5 immunocompetent 19- to 28-year-old men within 2 LAN gaming centers. This report highlights LAN gaming centers as potential hotspots for TB transmission and notes challenges faced when conducting contact-tracing investigations in such settings.

The outbreak timeline is shown in the online Technical Appendix Figure (<http://wwwnc.cdc.gov/EID/article/21/1/14-1159-Techapp1.pdf>). Patients A–D had frequented LAN center 1, but 3 months before patient A received a TB diagnosis, the center closed, and they continued their gaming activities at LAN center 2. Patient E had only patronized LAN center 2. In February 2012, the initial case-patient, patient A, sought medical care for a cough of 4 months' duration. Chest radiographs showed bilateral cavitory lesions, a sputum smear was positive for acid-fast bacilli, and a sputum culture grew *M. tuberculosis* with phenotypic resistance to rifampin, isoniazid, streptomycin, and ethionamide. Contact tracing for patient A was delayed because he defaulted on directly observed therapy after 5 days and eluded contact for 6 weeks.

Before his diagnosis, patient A had spent several hours daily at LAN center 1, where he participated in gaming and worked part time. Thirty contacts from center 1 were identified, but most failed to show up for screening until Ministry

of Health public health officers intervened 4 months after patient A received his diagnosis. The attack rate was 40% among the 30 contacts. Two contacts (patients B and C) were sputum TB culture-positive and had bacteria with identical drug-resistance phenotypes; 12 contacts were positive for latent TB (QuantiFERON-TB Gold In-Tube test; Cellestis Ltd, Carnegie, VIC, Australia). Patients D and E (not initially identified as patient A contacts) sought care for pulmonary TB 8 and 10 months, respectively, after patient A's diagnosis. Questioning revealed that they had patronized LAN center 2 during the same period as patients A–C. Culture results for patients D and E were positive for MDR TB, and bacteria had a drug-resistance phenotype identical to that for isolates from patients A–C.

Attempts to expand contact screening at LAN center 2 were met with resistance from the center's management. Thus, a nationwide alert was issued by the Ministry of Health, and legal orders and health advisories were served to LAN center employees and patrons, yielding 44 additional contacts, of whom, only 1 had positive results by the QuantiFERON-TB Gold In-Tube test. Contacts with latent TB infection were not treated because evidence is inconclusive for the efficacy of preventive therapy; these contacts had follow-up medical visits every 6 months for 2 years.

Results of spoligotyping (Ocimum Biosolutions, Hyderabad, India) and 24-loci mycobacterial interspersed repetitive units (MIRU) typing and variable number tandem repeats (VNTR) analysis (MIRU-VNTR Typing kit; GenoScreen, Lille, France) were identical for all 5 isolates; the isolates were shown to belong to the Beijing lineage (1). Our MIRU-VNTR database contains data for 112 *M. tuberculosis* isolates, representing 87.5% of 128 MDR TB cases diagnosed in Singapore during 2008–2012. Of these isolates, only 1 was identical to isolates in this investigation. That isolate derived from a karaoke lounge hostess (patient K) who received a diagnosis of pulmonary TB after a positive sputum smear in 2008 but left Singapore shortly after the diagnosis and was lost to follow-up.

Because MIRU-VNTR lacks discriminatory power for Beijing lineage isolates (2), we performed whole-genome sequencing (HiSeq 2000; Illumina, San Diego, CA, USA) on the 6 identical isolates to determine epidemiologic links. Paired-end reads were mapped to the H37Rv reference genome (GenBank accession no. NC000962.3) by using the Burrows–Wheeler aligner (3). Bioinformatics analysis and single-nucleotide polymorphism identification were performed as described (4,5). Isolates from patients A–E were identical, and that from patient K differed by 3 single-nucleotide polymorphisms (online Technical Appendix Figure). Questioning revealed that patient A had worked at the karaoke lounge when patient K was employed as a hostess,

but he did not recall encountering her. None of the other patients had visited the lounge and did not recall contact with patient K.

The proliferation of LAN gaming centers exemplifies how modern technology and urbanization have spawned new patterns of behavior and foci of TB transmission. In Asian countries, such centers are usually enclosed, air-conditioned spaces where patrons sometimes spend up to several hours each day, putting them at high risk for TB infection if 1 person among them is infectious. Contact-tracing investigations are challenging in such situations because contacts are not easily identified and may be reluctant to appear for screening. Investigation is difficult even within legal frameworks, such as that provided by Singapore's Infectious Diseases Act (6), which can be invoked to compel persons with an infectious disease and their contacts to submit to medical evaluation and treatment. Our investigation provides additional affirmation for the role of whole-genome sequencing in constructing a transmission chain, which in this outbreak enabled identification of the index patient.

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Serologic Assessment of Possibility for MERS-CoV Infection in Equids

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To the Editor: In 2012, a novel coronavirus termed Middle East respiratory syndrome coronavirus (MERS-CoV) emerged on the Arabian Peninsula; the virus has been responsible for >800 human cases. Recently, evidence of MERS-CoV infection in dromedaries was obtained from the Canary Islands, the Arabian Peninsula, and Africa (1–3). Viral sequences from dromedaries and from humans infected with MERS-CoV were highly similar, suggesting a prominent role of dromedaries as an animal reservoir of the virus (4). However, the serologic assessment of other animal species has been incomplete. Investigations of domestic animal species have been restricted to goats, sheep, and cattle (3) and a limited study of horses (n = 3) (5). No evidence of recent infection was found in either study.

Whereas most known CoVs have a highly restricted host range in vitro and in vivo, MERS-CoV has been found to infect a broad range of cell cultures derived from Old and New World camelids as well as humans, primates,

bats, pigs, and goats (6). MERS-CoV uses the receptor dipeptidyl-peptidase-4 (DPP-4) to enter its host cell (7). Sequence comparison between the receptor-binding domain of the MERS-CoV spike protein and several mammalian DPP-4 sequences showed a higher percentage identity in the amino acid residues critical for virus entry between human and horse DPP-4 than between human and dromedary DPP-4 (8). It has been shown that MERS-CoV can use horse DPP-4 expressed on nonsusceptible cells (9), but no data are available on susceptibility of primary horse cells. Therefore, members of the family *Equidae*, which include domestic horses, donkeys, and mules, might be susceptible to MERS-CoV infection. According to the Food and Agricultural Organization of the United Nations (<http://faostat.fao.org>), >800,000 equids (horses, mules, and donkeys) are present on the Arabian Peninsula, but their role as putative MERS-CoV animal reservoirs has not been investigated. Therefore, we assessed in vitro susceptibility of primary horse cells to MERS-CoV infection and searched for serologic evidence of infection with MERS-CoV in equids originating from Spain and the United Arab Emirates.

Primary cells derived from the kidney of 2 horses (termed PN-R and PFN-R) and an interferon-deficient primate cell line (VeroB4) were infected with MERS-CoV at a multiplicity of infection of 0.5 PFUs. Virus replication was quantified by real-time reverse transcription PCR (MERS-CoV *upE* assay) (10) and by plaque assay in Vero cells to confirm the production of infectious virus particles. Both cell lines showed clear cytopathic effects, an increase of viral RNA, and production of infectious virus progeny (Figure, panels A, B).

To investigate equids for signs of infection with MERS-CoV, we collected 1,053 serum samples from MERS-CoV–endemic and –nonendemic areas: 192 samples from adult

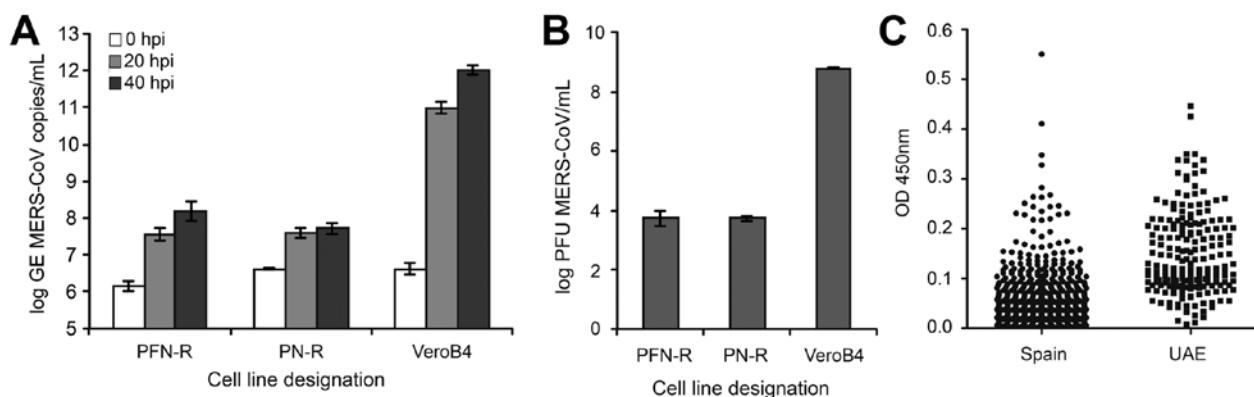


Figure. Analysis of the replication of Middle East respiratory syndrome coronavirus (MERS-CoV) in primary horse kidney cell lines and origin of equine serum samples. A, B) Cells were seeded at densities of 2×10^5 cells/mL and infected in triplicate with a multiplicity of infection of 0.5 infectious MERS-CoV units/cell. After incubation for 1 h, cells were washed twice and supernatants were harvested at 0, 20, and 40 h postinfection (hpi). The replication level is given as the log of the genome equivalents (A) or as PFUs (B). Error bars indicate SDs; PF-N and PFN-R indicate the 2 primary horse cell lines; VeroB4 is an interferon-deficient primate cell line. C) Distribution of optical density (OD) values (450 nm) of equine serum samples originating from Spain or the United Arab Emirates (UAE).

endurance horses from the United Arab Emirates that were collected for routine veterinary purposes; and 861 samples from 697 horses, 82 donkeys, and 82 mules in Spain. Because the reactivity of equid serum against MERS-CoV has not been investigated, we established a 2-stage algorithm for serologic testing that did not involve the determination of reactivity cutoff values. The screening stage involved testing of all serum samples by using a previously described ELISA with the spike protein S1-domain of MERS-CoV as the test antigen (4). The ELISA was adapted for use with horse serum by exchange of the secondary antibody. All serum samples reacted with low to medium OD values (range 0.0–0.55) (Figure, panel C). We then tested the 50 most reactive serum samples (optical density range 0.22–0.55) by using recombinant immunofluorescent and microneutralization assays (1). These assays are more specific than the ELISA assay and therefore can be used for confirmation. None of the tested serum samples showed reactivity in the recombinant immunofluorescent or microneutralization assays; this finding suggests that no previous exposure of equids to MERS-CoV has occurred in the United Arab Emirates and Spain.

Identifying all potential animal reservoirs is a critical step in controlling zoonotic diseases. Molecular data suggest that horses may be highly susceptible to MERS-CoV because of their high similarity in DPP-4 amino acids at positions critical for binding of the MERS-CoV spike protein (8). Our *in vitro* data confirm the susceptibility of primary horse cells, showing production not only of viral RNA but also of infectious virus progeny, which is a prerequisite for transmission. The lower replication observed in horse cells than in VeroB4 cells may be the result of a difference in the interferon competence of the cells; replication levels in horse cells are comparable to those in bat cells (6). Although we did not find evidence for equid infections with MERS-CoV in this study, the general susceptibility on the cell culture level suggests that equids from MERS-CoV–endemic areas, such as Africa and the Arabian Peninsula, should be further investigated for possible infection with MERS-CoV.

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Autochthonous Dengue Fever Imported to England from Japan, 2014

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To the Editor: Dengue fever, a mosquito-borne disease caused by dengue virus, can be asymptomatic or result in a variety of clinical manifestations, including fever, headache, myalgia, arthralgia, and rash (1). Severe cases can cause shock or severe hemorrhage (1). During the past 50 years, dengue has become a public health concern worldwide, rapidly spreading geographically, mainly in tropical and subtropical countries (1).

Before 2014, the most recent dengue outbreaks in Japan (located in the temperate zone), started in August 1942 on Kyusyu (the southernmost of Japan's 4 main islands) and recurred every summer until 1945 (2). Although no autochthonous dengue fever has been identified since then, a warning case occurred in August 2013, when dengue virus infection was diagnosed in a German traveler who returned from a 2-week trip to Japan (3). Reported here is an autochthonous dengue fever case imported from Japan to England in September 2014.

A 33-year-old international male student studying in England traveled to Japan and stayed with a friend in Tokyo during July–September 2014. In late August, an acute high fever (39.7°C), severe headache, retro-orbital pain, malaise, and loss of appetite developed. He did not have cough, sputum, or rhinorrhea. He returned to England 3 days after symptoms developed. On day 5 after symptom onset, the man noticed a scattered papular erythematous rash on the anterior chest wall. His symptoms had improved substantially. One week later, he experienced exacerbation of the same symptoms (again without cough, sputum, or rhinorrhea), which continued for a few days until he sought care 12 days after initial onset. He had no significant medical history or known allergies and had not traveled to any countries other than Japan.

On examination, the man appeared ill and had a high fever (38.5°C). His lungs were clear, but his pharynx was markedly swollen and erythematous with cervical lymphadenopathy. Despite his severe symptoms, results of laboratory tests showed no or only mild elevations: normal leukocyte count and differentials (6,700 cells/mm³ [reference 4,000–11,000] with neutrophils 60% and lymphocytes 26%) and slightly elevated C-reactive protein (0.9 mg/dL [reference 0.0–1.0 mg/dL]) and erythrocyte sedimentation rate (14 mm/h [reference 1–10 mm/h]). Liver enzyme levels were elevated: lactate dehydrogenase 623 IU/L (reference 125–243 IU/L), alanine aminotransferase 78 IU/L (reference 0–55 IU/L), aspartate aminotransferase 58 IU/L (reference 5–45 IU/L), and γ -glutamyl transpeptidase 81 IU/L (reference 12–64 IU/L), with normal total bilirubin (1.0 mg/dL [reference 0.2–1.2 mg/dL]). Mild hyponatremia (sodium 132 mmol/L [reference 135–145 mmol/L]) also was noted; otherwise the test results were unremarkable, including normal platelet count and coagulation panel. Serologic tests were positive for anti-dengue virus IgM but negative for anti-dengue virus IgG; viral RNA was not detected.

The patient was hydrated with intravenous fluid and discharged with acetaminophen as needed for fever and pain. He was instructed to avoid nonsteroidal antiinflammatory drugs because of possible bleeding risk. The sore throat resolved within 1 day, and his pain and fever were well controlled with acetaminophen.

This dengue outbreak in Japan started in Yoyogi Park, a public park in the Tokyo metropolitan area. From August

26, 2014, when the first case was identified, through October 30, a total of 160 autochthonous dengue fever infections occurred (4). The patient reported here stated that the house where he stayed in Japan is a 2-minute walk from Yoyogi Park and that he was bitten multiple times by mosquitoes in late August, although he did not enter the park or other implicated parks.

This outbreak has several possible causes. First, *Aedes albopictus* mosquitoes—1 of 2 main vector mosquitoes of dengue virus—are widespread in Japan. Although the other species, *A. aegypti*, has not been established in Japan, it was once identified at Tokyo International Airport (5). Second, the worldwide dengue fever incidence has increased exponentially during the past 50 years (1), and the number of cases imported to Japan has increased steadily since 1999 (6,7). Third, increased international travel, trade, and shipping, in addition to global warming, might have contributed to geographic expansion of the vectors.

Why this dengue fever outbreak started from Yoyogi Park remains unknown. One possibility is the popularity of Yoyogi Park, which holds \approx 100 events annually, including many international events. In July and August 2014, just before the first case was identified, Yoyogi Park hosted multiple festivals of countries in dengue-endemic regions, including Southeast Asia and South and Central America (<http://www.yoyogipark.info/ad2014/>). Dengue virus could have been spread from infected visitors by mosquitoes in the park. Any events that include persons from dengue-endemic regions and held where the vectors are prevalent could be the source of spread.

This case highlights the possible risk for a dengue outbreak in countries to which dengue is not endemic but where the vectors are present, and thus the potential exists for travelers to become infected. Physicians need to be aware of the possibility of dengue fever in patients returning from non-tropical/subtropical countries, obtain a full travel history, and keep apprised of the latest epidemic information.

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Genome Sequence of Enterovirus D68 from St. Louis, Missouri, USA

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To the Editor: During the current (2014) enterovirus/rhinovirus season in the United States, enterovirus D68 (EV-D68) is circulating at an unprecedented level. As of October 6, 2014, the Centers for Disease Control and Prevention (CDC) had confirmed 594 cases of EV-D68 infection in 43 states and the District of Columbia (<http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html>); the actual number of cases was undoubtedly much higher. In mid-August, hospitals in Missouri and Illinois noticed an increased number of patients with severe respiratory illness (1). We observed this pattern at St. Louis Children's Hospital in St. Louis, Missouri.

Resources for studying this virus are limited. Before the current season, only 7 whole-genome sequences and 5 additional complete coding sequences of the virus were available. Therefore, determining whether there are genomic elements associated with rapid spread or severe and unusual disease was not possible.

To address these limitations, we determined the complete coding sequence of 1 strain from St. Louis by using high-throughput sequencing of nucleic acid from a clinical sample. To evaluate the sequence diversity in EV-D68 strains circulating in the St. Louis metropolitan area, we also generated partial-genome sequences from 8 more EV-D68-positive clinical samples from St. Louis. During the

preparation of this article, CDC generated and submitted to GenBank 7 complete or nearly complete genome sequences from viruses obtained from the Midwest. We documented the diversity of the sequences of strains from St. Louis and compared them to publicly available sequences.

The methods are described in brief here and in more detail in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/1/14-1605-Techapp1.pdf>). This study was conducted under a protocol approved by the Human Research Protection Office of Washington University in St. Louis.

Patients were categorized retrospectively as having mild, moderate, or severe disease if they had been discharged home from the emergency unit, admitted to general wards, or admitted to the pediatric intensive care unit, respectively. Residual material from a subset of nasopharyngeal specimens positive for rhinovirus/enterovirus (tested by the BioFire FilmArray Respiratory Panel [BioFire Diagnostics, Salt Lake City, UT, USA] at the Clinical Virology Laboratory, St. Louis Children's Hospital) was selected for high-throughput sequencing. Total nucleic acid was extracted from clinical samples by using NucliSENS easyMAG (bioMérieux, Marcy l'Etoile, France) and used to make dual-indexed sequencing libraries. Enterovirus/rhinovirus sequences were enriched by using a NimbleGen custom sequence capture reagent (Roche/NimbleGen, Madison, WI, USA), which as of February 2014 was selective for all complete enterovirus and rhinovirus genomes in GenBank. Sequence data were generated on an Illumina HiSeq 2500 (Illumina Inc., San Diego, CA, USA). Sequences were assembled with IDBA-UD (2) and manually improved. The most contiguous genome was annotated by using VIGOR (3). Publicly available sequences were downloaded and compared by using the National Institute of Allergy and Infectious Diseases Virus Pathogen Resource (<http://www.viprbrc.org>) (4). Variants were identified by using VarScan (5). The sequence was deposited in GenBank under accession no. KM881710, BioProject PRJNA263037.

For 14 of the 17 samples, high-throughput sequencing data were interpretable (online Technical Appendix Table); for the other 3 samples, the number of virus sequence reads was too low to distinguish them from sample cross-talk, which occurs during high-throughput sequencing analysis (6). Of the 14 typed samples, EV-D68 sequences were detected in 7 of 10 samples from patients with severe disease, 2 of 2 with moderate disease, and 0 of 2 with mild disease. The complete coding sequence was assembled from sample EV-D68_STL_2014_12. The most closely related genomes from previous seasons were Thailand, CU134, and CU171 (7) (Figure, panel A). Several of the genome sequences obtained from Missouri strains from this season, which had been sequenced by CDC, were very similar to this genome sequence. Comparison of the virus protein 1 sequence with

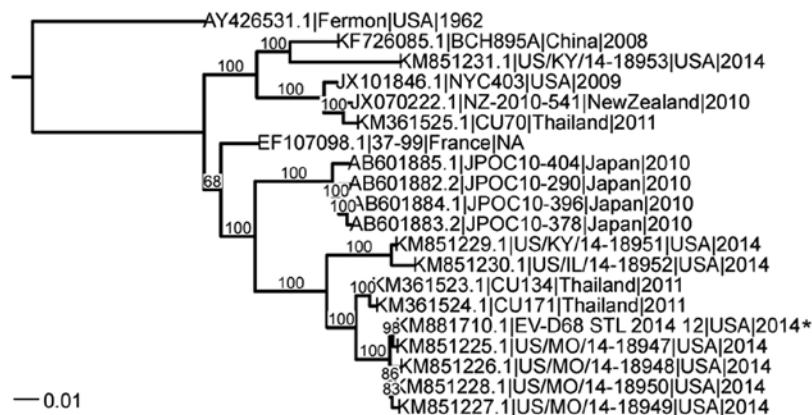
A**B**

Figure. Phylogenetic comparison of enterovirus D68 (EV-D68) obtained from St. Louis, Missouri, USA, in 2014, with other sequenced strains. The phylogenetic relationships of genome sequences (nucleotides) were estimated by using the maximum-likelihood method with RAxML (<http://www.viprbrc.org>) bootstrapped 100 times. A) Comparison of genome sequences for full-length and nearly full-length strains. B) Comparison of virus protein 1 sequences. Sequences were downloaded from ViPR (<http://www.viprbrc.org>) and supplemented with strains from the 2014 EV-D68 season. Sequences were clustered at 99% identity to obtain a representative set of sequences, which are shown. Bootstrap values are indicated on each tree. The strain from St. Louis is indicated by an asterisk on each tree. Scale bars indicate nucleotide substitutions per site.

that of publicly available sequences indicated that the strain from St. Louis and the strain from Missouri (CDC) cluster with virus strains identified in Europe and Asia within the past several years (Figure 1, panel B). The St. Louis virus shared 97%–99% aa sequence identity with all other sequenced strains. We observed little variation in the strains from St. Louis because they shared 98%–99% nt sequence identity (online Technical Appendix Figure).

We provide a genome sequence from the 2014 outbreak of EV-D68 infection in St. Louis, Missouri. This sequence seems to be highly representative of the strains circulating in St. Louis during this time because the other genomes we partially sequenced are very similar. To our knowledge, no amino acids have been associated with virulence or increased infectivity of EV-D68; therefore, we cannot associate the changes we observed in these genomes

to phenotypic traits. Because changes in the 5' untranslated region have the potential to affect the rate of replication (8–10), it is possible that minor genome changes are responsible for the rapid spread and high severity of disease in 2014. Correlation between clinical features of patients in conjunction with additional genomic analysis might provide further insight into the pathogenetic determinants of this strain. Therefore the genome sequence of EV-D68 determined from the 2014 outbreak in St. Louis, Missouri, provides a resource for tracking and genomic comparison of this rapidly spreading virus.

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Prisoners Treated for Hepatitis C with Protease Inhibitor, New York, USA, 2012

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To the Editor: Globally, epidemics of infection with hepatitis C virus (HCV) tend to be concentrated in correctional facilities, according to a review of worldwide literature during 1990–2012 (1,2). Nearly 1 in 3 persons infected with HCV in the United States spends at least part of each year in either a prison or jail (3). Proficiency by correctional health facilities in hepatitis C treatment by using the most effective agents may mitigate the predicted burden of end-stage liver disease and hepatocellular cancer in the coming years. The first wave of direct-acting agents against HCV had substantial side effects. Nonetheless, the New York Department of Corrections and Community Supervision (NYDOCCS) systematically approached the challenge of using these agents, and their experience serves as a lesson that prison medical services can overcome substantial barriers to care.

In 2011, NYDOCCS piloted a hepatitis C treatment program for HCV genotypes 1a and 1b, comprised of telaprevir, pegylated interferon, and ribavirin. Patients underwent extensive mandatory pretreatment screening for mental health issues, pregnancy, and cirrhosis by using the FibroSURE assay (LabCorp, Burlington, NC, USA) and Doppler sonogram of the portal vein. Eligibility requirements were a negative HIV test result, and liver fibrosis assessed either by liver biopsy or noninvasively, at METAVIR stage 2 or 3, or 4 (reference range 0–4) (4) with compensation. A patient in stage 1 was eligible if the patient had poor prognostic factors. An infectious disease consultant saw every patient; each patient received mandatory patient education. Mandatory conditions for patients participating in the program were to sign a consent

form agreeing to the medication, participate in laboratory monitoring, and attend primary or specialty clinic follow-up appointments; 3 refusals of any program component in any combination would result in medication discontinuation. Because referring physicians conducted the initial screening of potential participants, the exact number of patients screened cannot be determined. The most frequent factor for ineligibility was uncontrolled psychiatric disease.

Facilities were encouraged to treat ≤ 5 patients at any given time. Staff physicians, mid-level providers, nurses, pharmacists, and social workers participated in extensive training. Telaprevir was administered with a fat supplement by directly observed therapy. Patients waited in lines every 7–9 hours to receive the medications. All patients received antihistamine prophylaxis before therapy was initiated. Patients received containers of high potency topical steroid creams at the initiation of treatment with instructions to start applying it 2 times daily at the first sign of a rash. The nurses questioned the patients about rash and pruritus during each weekly interferon injection appointment. The infectious disease consultant conducted follow-up visits by telemedicine, using video and audio equipment for remote monitoring and supervision of treatment.

Among the 50 patients who began therapy, 38 (76%) were naïve to any treatment, 7 (14%) were experiencing relapses, 4 (8%) had responded partially to prior treatment, and 1 (2%) had not responded to prior treatment. Ages of the patients ranged from 21 to 67 years. All 50 treated patients experienced rash, pruritus, or both. All but 1 patient (98%) experienced fatigue. Anemia was detected in 68%, yet none received erythropoietin. Rather, the infectious disease consultant aggressively reduced ribavirin doses. Because of difficult-to-access, paper-based recording of dosing, determining the mean dose of ribavirin used in each patient was not feasible. None required transfusions. Thrombocytopenia developed in 2 patients. In 64%, neuropsychiatric effects were observed. Of 50 patients, 2 (4%) dropped out of the program. As shown in the Table, 44 (88%) experienced a sustained viral response.

By using the most effective agents available to treat hepatitis C, correctional health care systems can make a unique contribution to public health (5). Despite the substantial side effects of the first direct acting agents, the NYDOCCS health care personnel observed and reported a sustained viral response in a select group of patients which exceeded that seen in the drug registration trials (6). Recruiting a multidisciplinary health care team facilitated administration of a cumbersome regimen. Prophylactic treatment for rash and ribavirin dose reductions in 68% may have minimized dermatologic side effects. Mandatory patient education may have enhanced patient motivation to adhere to the protocol. As shown previously,

Table. Outcomes of 50 prisoners treated for hepatitis C within the New York State Department of Corrections and Community Supervision, New York, USA, 2012–2013*

Outcome	No. (%) prisoners	Metavir staging of fibrosis, no. (%) prisoners	
		Stage 1–3	Stage 4
Disenrolled	2 (4)	1 (2)	1 (11)
Treatment failed	4 (8)	2 (5)	2 (22)
Sustained viral response	44 (88)	38 (93)	6 (67)
Early rapid viral response	34 (68)	31 (76)	3 (33)
Total	50 (100)	41 (82)	9 (18)

*Treatment consisted of telaprevir, pegylated interferon, and ribavirin.

telemedicine was a valuable tool in hepatitis C management of prisoner-patients (7). Treatment administered by the NYDOCCS treatment team cured many HCV-infected patients. Correctional health providers can manage complex therapeutic protocols for treatment of voluntary participants in the controlled environment of these facilities.

Treatment with novel agents has ushered in a new era in hepatitis C management. The cost of newer drugs is daunting (e.g., \$84,000 for a 12-week course of sofosbuvir), but prison providers are starting to use this next generation of agents (8). Recent clinical trials with newer direct-acting agents that spare interferon have even higher rates of virologic success and fewer side effects (9,10) which should lead to even more widespread success.

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LETTERS

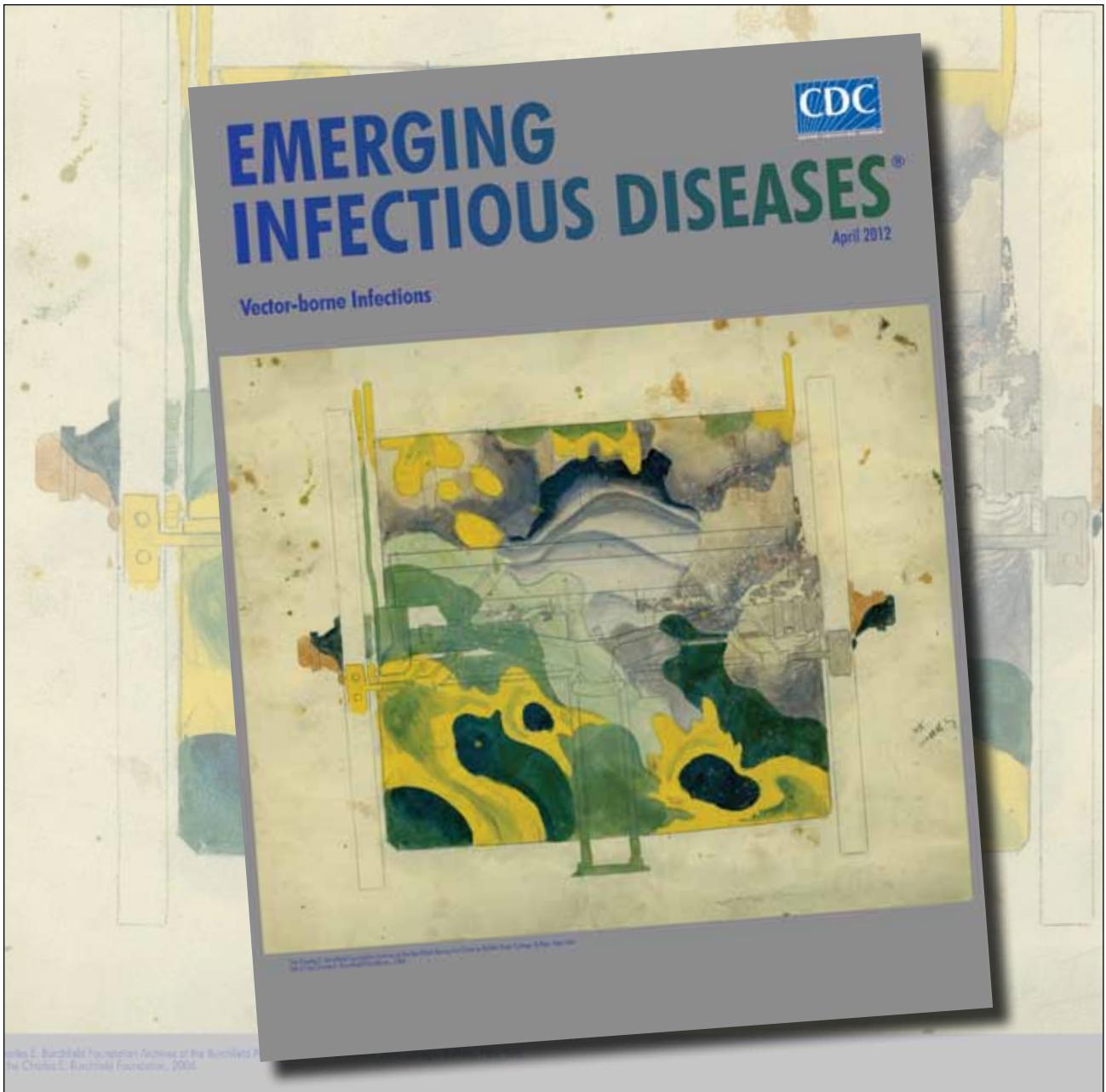
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Correction: Vol. 20, No. 3

The name of author Magnus Rasmussen was misspelled in the article Septic Arthritis Caused by *Streptococcus suis* Serotype 5 in Pig Farmer (C. Gustavsson, M. Ramussen). The article has been corrected online (http://wwwnc.cdc.gov/eid/article/20/3/13-0535_article).



CDC Health Information for International Travel 2014

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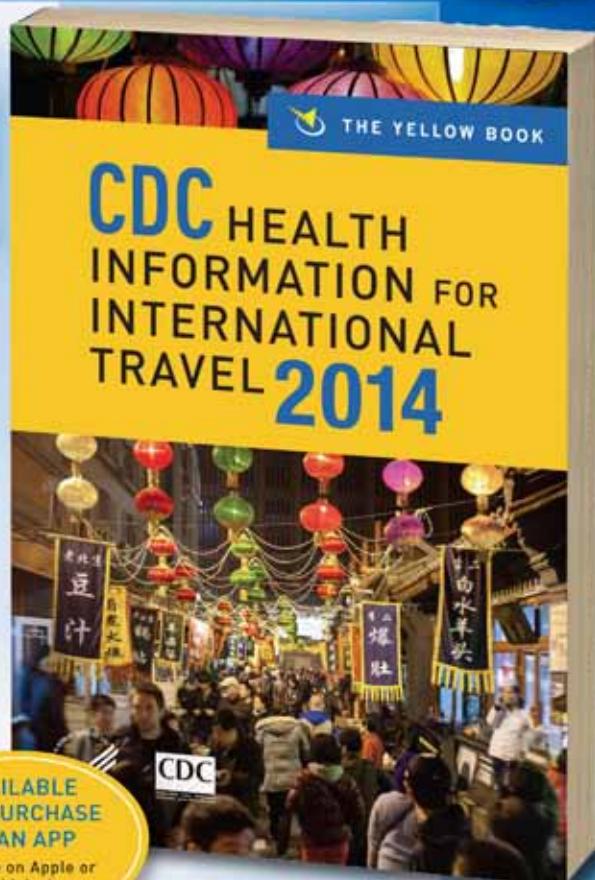
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ABOUT THE COVER



Rogan Brown (1972–) *Outbreak*, 2014. Hand cut paper, foam board, transparent domes (31.1 × 57.9 × 9.8 in / 79 × 147 × 25 cm)
©The artist/Image courtesy of the artist.

Delicacy and Durability: The Microbiological Sublime

Byron Breedlove

Anglo-Irish artist Rogan Brown, who creates monochromatic sculptures from layers of paper, considers *Outbreak*, this month's cover image, an exploration "of the microbiological sublime." Spilling from their petri dishes, overflowing into the space between them, the organisms appear kinetic; the sweep of light and shadow across the image adds depth and dimension to the all-white forms. For full impact, though, this sculpture needs to be viewed in person. In the artist's words, "Each piece suddenly comes alive when it is placed vertically in the light. Photos only catch them at a certain moment. In reality, the pieces move with the changes in the ambient lighting, so they are always slightly different." (The quoted text in this essay is from personal communication with Rogan Brown, November 17, 2014.)

To prepare for his work on *Outbreak*, Brown studied myriad photos and diagrams depicting bacteria and viruses.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

"For the bacteria, I was attracted more by the flagellate forms (salmonella, *E. coli*, *Vibrio cholerae*) simply because they offer a more aesthetically interesting bug-like shape, both beautiful and creepy at the same time, which was the effect I wanted to create. For the viruses, I was looking at influenza and HIV because these are forms that are most accessible and familiar to the public." He notes that "Although I reference the aesthetics of scientific illustration, diagrams, cut-away models, and so forth, my goal is not to create accurate representations but works of art that create a visual, sensual impact."

Pathogens and microbes vary immensely in their form, color, and complexity. Stains and dyes are often used to highlight pathogens viewed through microscopes, and those colored, contrasted images may be the first to come to mind. Instead of color, however, Brown focuses on contour and shape, methodically sculpting sheets of paper. For that task, he relies on both traditional tools such as knives and scalpels and modern tools such as laser cutters.

He cites the gluing process as being more stressful than cutting: “Each layer has to be placed with perfect precision on top of the preceding one. There are usually about 8 layers of paper separated by a hidden spacer to create the illusion of floating. The glue does not allow repositioning. I have only one shot, and mistakes are sometimes made.” Brown, who sometimes spends up to 5 months on a project, explains that “The finished artefact is really only the ghostly fossilized vestige of this slow, long process I have chosen paper as a medium because it captures perfectly that mixture of delicacy and durability that for me characterizes the natural world.”

Originally, Brown planned to create a large installation from which the pathogens would swarm beyond the frame and flow over its walls, spilling onto floor of the gallery space—an idea that may yet see fruition. He found inspiration for this sculpture while attending a microbiology seminar at the Eden Project, a visitor attraction in the United Kingdom; during this seminar, a planned exhibition space focused on the Human Microbiome Project was discussed.

High-consequence pathogens, several of which are highlighted in this issue of *Emerging Infectious Diseases*, provide a rich vein of inspiration for fiction writers and filmmakers. Actual outbreaks caused by such pathogens

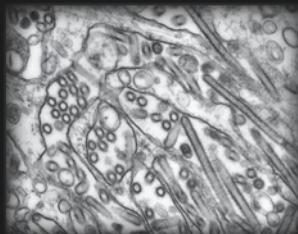
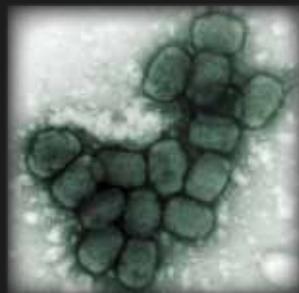
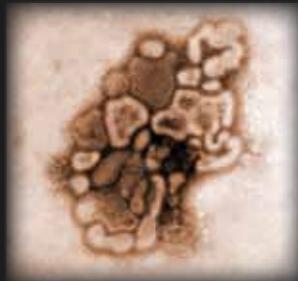
attract feverish media attention. Brown explains, “I wanted to create this sense of ferocious energy bursting out of the petri-dome, mocking our attempts to control it. Although created before the recent Ebola outbreak, the installation plays on our fears of the microbiological and our sense of powerlessness when confronted by nature.” The frozen tension inherent in *Outbreak* plays to the imagination, starkly capturing the potential genesis of a frightening occurrence.

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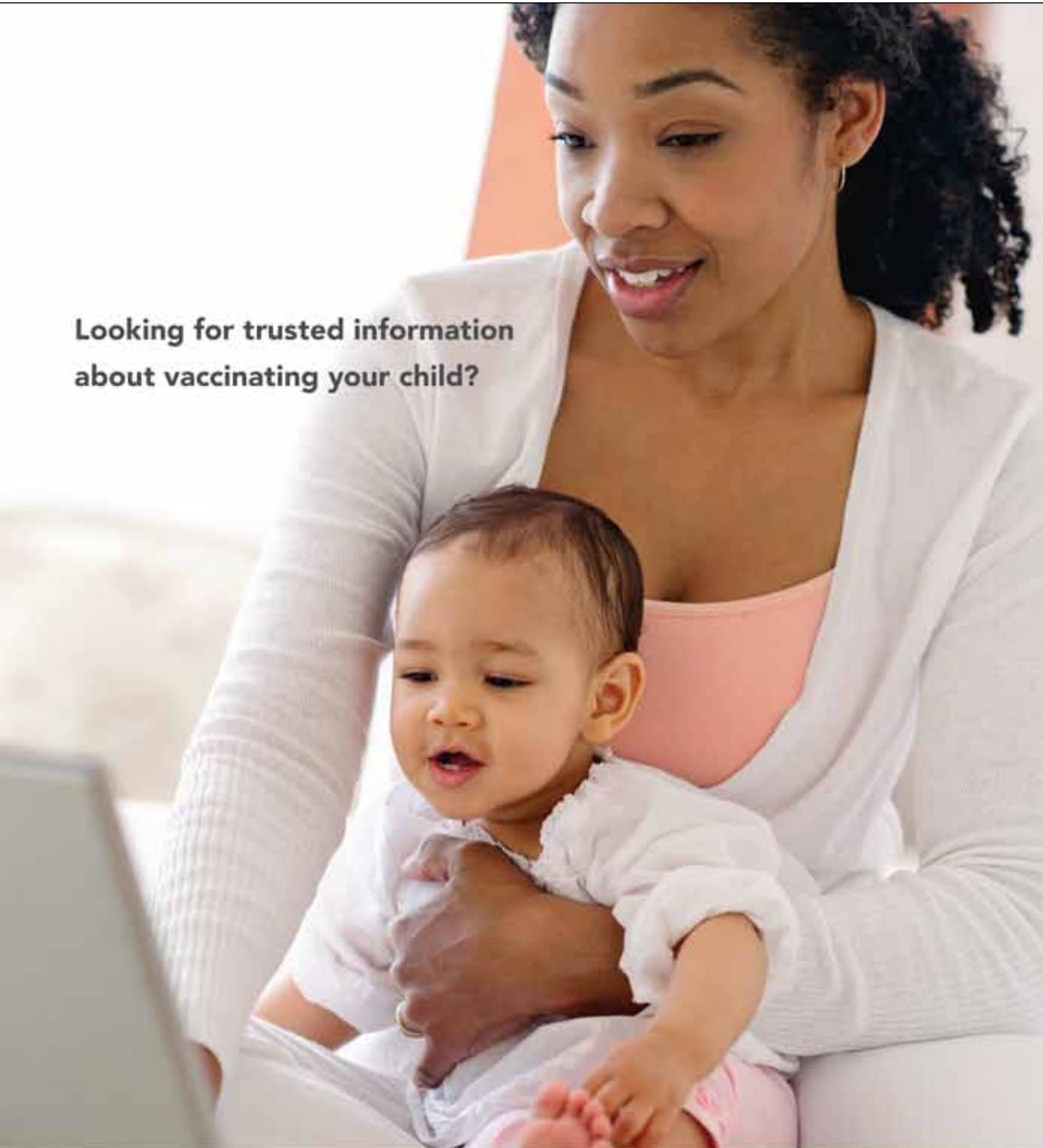


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Upcoming Issue

Evaluation of Border Entry Screening for Infectious Diseases in Humans

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

Optimizing Distributions of Pandemic Influenza Antiviral Drugs

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

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

Tickborne Relapsing Fever, Bitterroot Valley, Montana, USA

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

Quantifying Reporting Timeliness to Improve Outbreak Control

Encephalitis and Meningoencephalitis in Thailand, 2003–2005

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

Neisseria meningitidis ST-11 Clonal Complex, Chile 2012

Acquisition of Human Polyomaviruses in the First 18 Months of Life

Cluster of Middle East Respiratory Syndrome Coronavirus Infections in Iran, 2014

Vesicular Stomatitis Virus–based Vaccines against Lassa and Ebola Viruses

Outbreak of Henipavirus Infection, Philippines, 2014

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

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

Comparative Analysis of African Swine Fever Virus Genotypes and Serogroups

Close Relationship between West Nile Virus from Turkey and Lineage 1 Strain from Central African Republic

Potentially Novel *Ehrlichia* Species in Horses, Nicaragua

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

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

Upcoming Infectious Disease Activities

2015

February 23–25, 2015

CROI

Conference on Retroviruses and Opportunistic Infections
Seattle, WA, USA

<http://www.croi2014.org>

April 20–23, 2015

EIS

Epidemic Intelligence Service
Conference

<http://www.cdc.gov/eis/conference.html>

May 14–17, 2015

SHEA

The Society for Healthcare
Epidemiology of America
Orlando, FL, USA

<http://shea2015.org/attendees/registration/>

May 30–June 2, 2015

American Society for Microbiology
General Meeting
New Orleans, LA, USA

<http://gm.asm.org/>

August 24–28, 2015

ICEID

International Conference
on Emerging Infectious Diseases
Atlanta, GA, USA

August 29–September 2, 2015

IDBR

20th Annual Infectious Disease
Board Review Course
McLean, VA, USA

<http://smhs.gwu.edu/cehp/activities/courses/idbr>

2016

March 2–5, 2016

ISID

17th International Congress
on Infectious Diseases
Hyderabad, India

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

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Article Title

Epidemiology of Human Plague in the United States, 1900–2012

CME Questions

1. You are seeing a 30-year-old man with a 3-day history of a mass in his right groin along with fever and chills. Bubonic plague is on your list of differential diagnoses as you evaluate him. According to the current study by Kugeler and colleagues, which of the following statements regarding the epidemiology of plague is most accurate?

- A. Cases of plague have been reported in all 50 states
- B. Most cases occur among adults older than 60 years
- C. Most cases were reported among African Americans
- D. Nearly two-thirds of cases occurred among men

2. What was the most common primary clinical form of plague in the current study by Kugeler and colleagues?

- A. Bubonic
- B. Pneumonic
- C. Septicemic
- D. Gastrointestinal

3. What should you consider regarding temporal trends in the prevalence of plague in the current study by Kugeler and colleagues?

- A. Cases were originally widespread throughout the United States, and then focused in certain regions only
- B. Across time, cases became exclusively limited to the East Coast of the United States
- C. Less than 2% of cases occurred after 1965
- D. There have been at least some cases of plague reported annually between 1965 and 2012

4. Results on ancillary testing for this patient confirm a diagnosis of bubonic plague. Which of the following statements regarding the survival outcomes of plague in the current study by Kugeler and colleagues is most accurate?

- A. Mortality rate decreased to near 0% after the advent of appropriate antibiotic therapy
- B. Antibiotics have a positive effect in improving survival
- C. Bubonic plague is the primary clinical form of plague associated with the highest risk for mortality
- D. Pharyngeal plague is very rarely fatal

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree

1

2

3

4

Strongly Agree

5

2. The material was organized clearly for learning to occur.

Strongly Disagree

1

2

3

4

Strongly Agree

5

3. The content learned from this activity will impact my practice.

Strongly Disagree

1

2

3

4

Strongly Agree

5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree

1

2

3

4

Strongly Agree

5

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to <http://www.medscape.org/journal/eid>. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to <http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page>. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Clinical Course and Long-Term Outcome of Hantavirus-Associated Nephropathia Epidemica, Germany

CME Questions

1. Your patient is a 52-year-old man with Puumala virus (PUUV) hantavirus infection. According to the cross-sectional prospective survey by Latus and colleagues, which of the following statements about the clinical characteristics of acute nephropathia epidemica (NE) associated with PUUV hantavirus infection is correct?

- A. Back pain, limb pain, and visual disorders are unusual during acute NE
- B. An estimated 86% of patients had acute kidney injury (AKI) by RIFLE criteria
- C. Most patients with AKI required transient renal replacement therapy
- D. Thrombocytopenia occurred in one fifth of patients

2. According to the cross-sectional prospective survey by Latus and colleagues, which of the following statements about long-term sequelae of acute NE associated with PUUV hantavirus infection is correct?

- A. At follow-up, 23% of patients had hypertension, of whom one third had preexisting hypertension and 67% had newly diagnosed hypertension
- B. Proteinuria is often a long-term consequence of NE

- C. Hematuria is not a long-term consequence of NE
- D. The incidence of high blood pressure was significantly higher than in an age-matched cohort of patients without any history of NE

3. According to the cross-sectional prospective survey by Latus and colleagues, which of the following statements about serologic findings of NE associated with PUUV hantavirus infection would most likely be accurate?

- A. Presence of neutralizing antibodies at long-term follow-up was explained by recurrent disease
- B. At follow-up, one quarter of patients had detectable hantavirus-specific immunoglobulin G (IgG)
- C. At follow-up, one quarter of patients had persistent IgM antibodies
- D. Hantavirus-specific IgG and IgM were detected at time of diagnosis of NE

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	

Ticks and Lyme Disease



For more information about Lyme disease, visit <http://www.cdc.gov/Lyme>

How to prevent tick bites when hiking and camping

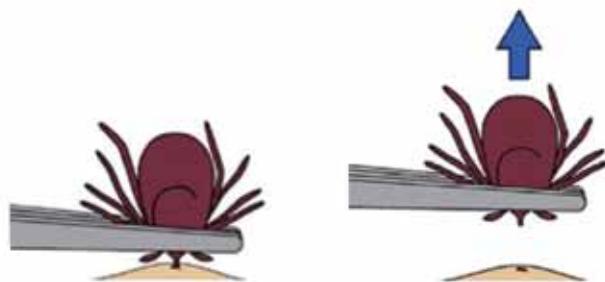
Ticks can spread disease, including Lyme disease. Protect yourself:

- Use insect repellent that contains 20 - 30% DEET.
- Wear clothing that has been treated with permethrin.
- Take a shower as soon as you can after coming indoors.
- Look for ticks on your body. Ticks can hide under the armpits, behind the knees, in the hair, and in the groin.
- Put your clothes in the dryer on high heat for 60 minutes to kill any remaining ticks.

How to remove a tick

1. If a tick is attached to you, use fine-tipped tweezers to grasp the tick at the surface of your skin.
2. Pull the tick straight up and out. Don't twist or jerk the tick—this can cause the mouth parts to break off and stay in the skin. If this happens, remove the mouth parts with tweezers if you can. If not, leave them alone and let your skin heal.
3. Clean the bite and your hands with rubbing alcohol, an iodine scrub, or soap and water.
4. You may get a small bump or redness that goes away in 1-2 days, like a mosquito bite. This is not a sign that you have Lyme disease.

Note: Do not put hot matches, nail polish, or petroleum jelly on the tick to try to make it pull away from your skin.



If you remove a tick quickly (within 24 hours) you can greatly reduce your chances of getting Lyme disease.

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.

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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.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of sub-headings 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.

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Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.