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Transmission of *Mycobacterium tuberculosis* Beijing Strains, Alberta, Canada, 1991–2007

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Learning Objectives

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

- Describe the epidemiology and previous research of the Beijing strains
- Distinguish characteristics of pulmonary TB with the Beijing strains in the current study
- Compare the transmissibility of the Beijing and non-Beijing strains in the current study
- Analyze factors associated with secondary cases of pulmonary TB in the current study

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Beijing strains are speculated to have a selective advantage over other *Mycobacterium tuberculosis* strains because of increased transmissibility and virulence. In Alberta, a province of Canada that receives a large number of immigrants, we conducted a population-based study to determine whether Beijing strains were associated with increased transmission leading to disease compared with

non-Beijing strains. Beijing strains accounted for 258 (19%) of 1,379 pulmonary tuberculosis cases in 1991–2007; overall, 21% of Beijing cases and 37% of non-Beijing cases were associated with transmission clusters. Beijing index cases had significantly fewer secondary cases within 2 years than did non-Beijing cases, but this difference disappeared after adjustment for demographic characteristics, infectiousness, and *M. tuberculosis* lineage. In a province that has effective tuberculosis control, transmission of Beijing strains posed no more of a public health threat than did non-Beijing strains.

The Beijing lineage of *Mycobacterium tuberculosis* (also referred to as the East Asian lineage or lineage 2) is an emerging public health threat (*1*,*2*). The Beijing lineage accounts for 13% of *M. tuberculosis* strains globally (*3*) and 19%–27% of *M. tuberculosis* strains in low tuberculosis (TB) incidence immigrant-receiving countries, such as Australia, the United States, and Canada (*4*–*6*). In addition to their recent global dissemination, Beijing lineage strains raise concern because of frequent associations with drug resistance and multidrug-resistant TB in particular (*1*,*6*–*8*). Reports of Beijing strains that are extensively drug resistant further intensify these concerns (*7*).

The rapid global expansion of Beijing strains and their frequent (albeit inconsistent) association with large TB outbreaks and younger patients has led to speculation that Beijing strains have a selective advantage over other *M. tuberculosis* lineages as conferred through increased transmissibility and virulence (*1*,*2*,*7*). This hypothesis is supported by experimental evidence of the increased virulence of Beijing lineage strains relative to other *M. tuberculosis* strains in vitro and in animal models (*9*,*10*). Evidence also suggests that the fitness of some Beijing strains is retained after the acquisition of drug resistance (*11*). Nevertheless, intragenotypic variation in virulence has been described in the Beijing family (*12*,*13*) and, in a review, Coscolla and Gagneux (*14*) concluded that the current body of evidence is insufficient to support the increased transmissibility of these strains.

Immigration is the main determinant of TB epidemiology in low incidence settings (*15*,*16*). Consequently, the importation of potentially more pathogenic strains, such as those in the Beijing family, could have major implications for TB prevention and elimination efforts within immigrant-receiving countries. Surveillance activities that identify the sources and transmission patterns of emerging and/or expanding *M. tuberculosis* strains will be increasingly vital if TB prevention and care programs are to maintain their effectiveness within the context of dynamic immigration policies and highly mobile populations.

We aimed to investigate the association of Beijing and non-Beijing lineage strains with transmission in a low TB incidence immigrant-receiving province of Canada. In particular, we sought to determine whether the Beijing lineage of *M. tuberculosis* is a greater public health threat than other strains because of increased transmission leading to disease.

Methods

Study Setting and Population

Culture-confirmed pulmonary TB cases (potential transmitters) in the province of Alberta, Canada, during January 1, 1991–June 30, 2007 (i.e., study period) in accordance with the provincial TB registry were eligible for inclusion in this population-based retrospective study (see Transmission Leading to Disease below for additional criteria). These cases represent the pulmonary subset of previously reported cases (*6*). Ethics approval was received from the University of Alberta Health Research Ethics Board, and analysis of surveillance data did not require informed consent because there was no direct patient contact.

Persons born in Canada or born outside of Canada to Canadian-born parents were considered Canadian-born; all others were foreign-born. The Canadian-born population was not further categorized into Aboriginal and non-Aboriginal groups because only 5 Beijing TB cases occurred among Aboriginal peoples during the study period (*6*). However, because of the high prevalence of Beijing strains in parts of Southeast and East Asia (*1*), country of birth was used to group foreign-born persons into those born in the Western Pacific region and those born elsewhere (*16*).

Case Characteristics

Demographic and clinical data from the TB Registry were combined with data from the Provincial Laboratory for Public Health (Provincial Laboratory). The Provincial Laboratory conducts all mycobacteriology studies in the province in accordance with the Canadian Tuberculosis Standards (*17*).

Sputum smear status and the presence or absence of lung cavitation on chest radiograph were used as indicators of infectiousness. Baseline sputum smears collected on, or within the week before, the date of diagnosis (the start date of treatment) that had grade 3+ to 4+ scores for acid-fast bacilli were categorized as having high bacillary loads (*17*). Monoresistant isolates had resistance to a single first-line drug, namely isoniazid, rifampin, pyrazinamide, ethambutol, or streptomycin (*17*). Resistance to ≥2 first-line drugs but without isoniazid–rifampin resistance constituted polyresistance, whereas multidrug-resistant TB comprised cases with resistance to at least isoniazid–rifampin.

The Provincial Laboratory completed DNA fingerprinting of prospectively archived isolates with the IS*6110*

restriction fragment-length polymorphism (RFLP) method, and for isolates with ≤5 copies of IS*6110*, spoligotyping was performed as described (*18*). Isolates were also assigned to an *M. tuberculosis* lineage at the Provincial Laboratory according to the PCR-based detection of large sequence polymorphisms as described (*19*,*20*). Isolates with a deletion of RD105 were classified as Beijing lineage strains and all others as non-Beijing lineage strains.

Transmission Leading to Disease

Of the 1,399 eligible pulmonary TB cases during the study period, 20 (1%) were excluded because either the DNA fingerprint pattern or *M. tuberculosis* lineage could not be determined. The remaining 1,379 cases were included in an analysis of clustering to provide an indication of overall transmission leading to disease during the study period. A cluster was defined as ≥2 patients whose case isolates had identical DNA fingerprints.

In addition to overall transmission, recent transmission that led to disease was quantified to account for dissimilarities in the follow-up periods of potential source cases and to minimize the probability of propagated transmission by second and later generation source cases (*21*). A Kaplan-Meier survival analysis was completed with the 1,379 pulmonary TB cases to determine the cutoff point for the definition of recent transmission (*21*,*22*). The Kaplan-Meier probability of an isolate being followed by another isolate with an identical fingerprint pattern during the 16.5-year study period was 0.36; the probability of ≥2 isolates having identical fingerprint patterns in a 2- and 3-year period was 0.22 and 0.24, respectively (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1578-Techapp1.pdf). Given the similarity in these latter probabilities, the 2-year period was subsequently determined to be the ideal cutoff period because it coincided with the conventional high-risk period for the development of active TB after recent infection (18–24 months) (*17*).

Using the 2-year cutoff point, we defined an index case as a pulmonary TB case with a DNA fingerprint pattern that had not been assigned to another case within the preceding 2 years. A secondary case was any case that had an identical fingerprint pattern as an index case provided that it was also diagnosed no more than 2 years after the index case. Using these definitions, we excluded 430 (31%) of 1,379 TB cases from the analysis of recent transmission. Specifically, 167 index cases diagnosed during 1991–1992 and their 50 secondary cases were excluded because we could not determine whether the fingerprint patterns of the index cases matched another case in the preceding 2 years. Follow-up periods of <2 years resulted in the exclusion of an additional 124 index cases diagnosed after June 2005 and their 10 secondary cases. Finally, 79 secondary cases were excluded because of diagnosis >2 years after the index case but <2 years after another cluster member. After these exclusions, 949 (69%) cases diagnosed during January 1, 1993–June 30, 2007, were included in the primary analysis of recent transmission.

Statistical Analysis

We analyzed data using Stata/IC 11 (StataCorp LP, College Station, TX, USA). For overall transmission, associations between case characteristics and *M. tuberculosis* lineage were assessed with bivariate and multivariate logistic regression analyses at a 5% level of significance. Characteristics of case-patients (sex, age at diagnosis, population group, sputum smear status, bacillary load, lung cavitation, drug resistance and clustering) that were $p<0.2$ in bivariate analyses were eligible for inclusion in the multivariate model. Subgroup analyses were also completed to evaluate intragenotypic associations between clustering and case-patient characteristics by using bivariate and multivariate logistic regression analyses.

For the analyses of recent transmission, transmission indices were calculated as the total number of secondary cases within the cutoff period divided by the total number of index cases (*21*). The risk factors of index cases that were associated with recent transmission leading to disease (i.e., relative transmission indices) were assessed with bivariate and multivariate Poisson regression by using an offset of 1 for each index case (*21*). Specifically, associations with sex, age at diagnosis, population group, sputum smear status, bacillary load, lung cavitation, drug resistance, and *M. tuberculosis* lineage were initially analyzed with bivariate Poisson regression. Multivariate Poisson regression modeling was constructed with purposeful selection and, with the exception of *M. tuberculosis* lineage, variables that had $p<0.20$ in bivariate regression were included in the initial multivariate model. As the primary variable of interest in this study, *M. tuberculosis* lineage was included in all multivariate models regardless of its significance.

For all multivariate regression modeling, the confounding effects of removed variables ($p\geq0.05$) were assessed with the percentage rule. We used a collapsibility criterion ≤15%, and the significance of potential interactions was based on the partial likelihood ratio test (*23*).

We assessed the influence of the length of the cutoff period on recent transmission with sensitivity analyses using 3- and 5-year cutoff periods. Additional analysis was completed with no cutoff period, the index case for each fingerprint pattern being the isolate in the dataset with the earliest date of diagnosis. We also evaluated the potential effect of including nonpulmonary secondary cases in the analysis of recent transmission. For this latter analysis, all nonpulmonary TB cases registered in Alberta during the study period that had an identical DNA fingerprint as a pulmonary index case were eligible for study inclusion.

Results

We identified Beijing strains in 258 (19%) of the 1,379 pulmonary TB cases in 1991–2007. Compared with non-Beijing cases, Beijing cases occurred among persons of similar sex and age but more often foreign-born (p<0.0001) (Table 1). The infectiousness of Beijing and non-Beijing cases was similar in relation to sputum smear status, bacillary load, and presence/absence of lung cavitation (Table 1). *M. tuberculosis* lineage and drug resistance were not independently associated (Table 1).

Overall Transmission

Overall, 906 (66%) cases exhibited unique fingerprint patterns (nonclustered cases), and 473 (34%) clustered cases were dispersed among 119 clusters. Of Beijing cases, 203 (79%) were nonclustered, and 55 (21%) were distributed among 22 clusters. Non-Beijing cases accounted for the remaining 703 (63%) nonclustered cases and 418 (37%) clustered cases within 97 clusters. Beijing cases were half as likely as non-Beijing cases to be clustered (p<0.0001), but this difference disappeared after we controlled for demographic characteristics, infectiousness, and drug resistance (p = 0.405) (Table 1).

Intragenotypic analysis showed that the clustering of Beijing cases was not associated with demographic characteristics, infectiousness, or drug resistance (Table 2). In contrast, the likelihood of non-Beijing cases being clustered was significantly less when patients were >64 years of age at diagnosis (vs. <35 years; p<0.0001) or foreign-born (p<0.0001) (Table 2). Although resistance to a single first-line anti-TB drug appeared to be associated with less clustering in bivariate analysis (p = 0.001), it was not associated with clustering independent of sex, age at diagnosis, and population group (p = 0.102) (Table 2).

In each lineage group, the number of nonclustered TB cases in foreign-born persons was inversely associated with time since arrival, such that 30%–32% of these cases occurred within the first 2 years after arrival (Figure). Clustered cases appeared to follow a similar pattern (Figure), although interpretation was limited by the relatively small number of cases.

Recent Transmission

Cases excluded from the analysis of recent transmission were demographically and clinically similar to included cases (data not shown). On average, an index case

Table 1. Characteristics of persons with pulmonary TB associated with *Mycobacterium tuberculosis* Beijing and non-Beijing strains, Alberta, Canada, 1991–2007

Characteristic	Strain		OR (95% CI)*	
	Beijing, no. (%)	Non-Beijing, no. (%)	Unadjusted	Adjusted
Sex				
F	100 (38.8)	492 (43.9)	1.0	1.0
M	158 (61.2)	629 (56.1)	1.2 (0.9–1.6)	1.3 (0.9–1.8)
Age at diagnosis, y				
<35	72 (27.9)	319 (28.5)	1.0	1.0
35–64	69 (26.7)	421 (37.6)	0.7 (0.5–1.04)	0.7 (0.1–1.01)
>64	117 (45.3)	381 (34.0)	1.4 (0.98–1.9)	1.1 (0.7–1.6)
Population group				
Canadian-born	19 (7.4)	520 (46.4)	1.0	1.0
Foreign-born, other	26 (10.1)	346 (30.9)	**2.1 (1.1–3.8)**	**2.1 (1.1–4.1)**
Foreign-born, Western Pacific	213 (82.6)	255 (22.7)	**22.9 (14.0–37.4)**	**22.6 (13.1–39.2)**
Sputum smear status†				
Negative	132 (54.1)	517 (49.0)	1.0	1.0
Positive	112 (45.9)	538 (51.0)	0.8 (0.6–1.1)	1.2 (0.8–1.7)
Bacillary load‡§				
Low	38 (70.4)	177 (66.5)	1.0	
High	16 (29.6)	89 (33.5)	0.8 (0.4–1.6)	
Results of chest radiography				
No cavitation	206 (79.8)	853 (76.1)	1.0	1.0
Cavitation	52 (20.2)	268 (23.9)	0.8 (0.6–1.1)	0.8 (0.5–1.3)
Drug resistance				
Pan-susceptible	202 (78.3)	1000 (89.2)	1.0	1.0
Monoresistance	30 (11.6)	90 (8.0)	**1.7 (1.1–2.6)**	0.8 (0.5–1.3)
Polyresistance	20 (7.8)	25 (2.2)	**4.0 (2.2–7.3)**	1.7 (0.9–3.5)
Multidrug resistance	6 (2.3)	6 (0.5)	**5.0 (1.6–15.5)**	3.7 (0.9–14.7)
Clustering				
Nonclustered	203 (78.7)	703 (62.7)	1.0	1.0
Clustered	55 (21.3)	418 (37.3)	**0.5 (0.3–0.6)**	0.8 (0.6–1.3)
Total	258 (100.0)	1,121 (100.0)		

*__Boldface__ indicates significance (p<0.05). TB, tuberculosis; OR, odds ratio.
†Sputum smear microscopy was not completed for all cases.
‡Semiquantitative scores for acid-fast bacilli load on the baseline sputum smear. Positive smears with semiquantitative scores of 3+ or 4+ were categorized as having high bacillary load; all remaining positive smears were labeled as having a low bacillary load.
§Bacillary load was not included in multivariate modeling because of multicollinearity with sputum smear status.

resulted in 0.13 secondary cases within 2 years. In unadjusted analysis, the number of secondary cases was higher if the index case-patient was sputum smear positive, had a high bacillary load, or had lung cavitation (Table 3). Conversely, fewer secondary cases were associated with index case-patients who were >64 years of age (vs. <35 years), foreign-born, or infected with Beijing strains. In adjusted analysis, the number of secondary cases was associated with the age, population group, and smear status of the index case-patients (Table 3). Specifically, fewer secondary cases occurred if the index case-patient was >64 years of age (vs. <35 years) or foreign-born whereas an increased

Table 2. Case-patient characteristics associated with clustering of Beijing and non-Beijing *Mycobacterium tuberculosis* lineages, Alberta, Canada, 1991–2007

Characteristic	All cases	No. (%) clustered cases	OR (95% CI)*	
			Unadjusted	Adjusted†
Beijing				
Sex				
F	100	19 (19.0)	1.0	
M	158	36 (22.8)	1.3 (0.7–2.3)	
Age at diagnosis, y				
<35	72	17 (23.6)	1.0	1.0
35–64	69	19 (27.5)	1.2 (0.6–2.6)	1.3 (0.6–2.8)
>64	117	19 (16.2)	0.6 (0.3–1.3)	0.7 (0.3–1.5)
Population group				
Canadian-born	19	7 (36.8)	1.0	
Foreign-born, other	26	6 (23.1)	0.5 (0.1–1.9)	
Foreign-born, Western Pacific	213	42 (19.7)	0.4 (0.2–1.1)	
Sputum smear status				
Negative	132	26 (19.7)	1.0	
Positive	112	25 (22.3)	1.2 (0.6–2.2)	
Bacillary load				
Low	38	7 (18.4)	1.0	
High	16	6 (37.5)	2.7 (0.7–9.8)	
Chest radiography				
No cavitation	206	40 (19.4)	1.0	1.0
Cavitation	52	15 (28.8)	1.7 (0.8–3.4)	1.6 (0.8–3.2)
Drug resistance				
Pan-susceptible	202	42 (20.8)	1.0	
Monoresistance	30	7 (23.3)	1.2 (0.5–2.9)	
Polyresistance	20	6 (30.0)	1.6 (0.6–4.5)	
Multidrug resistance	6	0	NA	
Non-Beijing				
Sex				
F	492	168 (34.1)	1.0	1.0
M	629	250 (39.7)	1.3 (0.98–1.6)	1.1 (0.8, 1.4)
Age at diagnosis, y				
<35	319	134 (42.0)	1.0	1.0
35–64	421	182 (43.2)	1.1 (0.8–1.4)	0.8 (0.5–1.1)
>64	381	102 (26.8)	**0.5 (0.4–0.7)**	**0.4 (0.3–0.6)**
Population group				
Canadian-born	520	316 (60.8)	1.0	1.0
Foreign-born, other	346	42 (12.1)	**0. 1 (0.06–0.13)**	**0.09 (0.06–0.13)**
Foreign-born, Western Pacific	255	60 (23.5)	**0.20 (0.14–0.28)**	**0.21 (0.15–0.31)**
Sputum smear status				
Negative	517	198 (38.3)	1.0	
Positive	538	199 (37.0)	0.9 (0.7–1.2)	
Bacillary load				
Low	177	67 (37.9)	1.0	
High	89	39 (43.8)	1.3 (0.8–2.1)	
Chest radiography				
No cavitation	853	312 (36.6)	1.0	
Cavitation	268	106 (39.6)	1.1 (0.9–1.5)	
Drug resistance				
Pan-susceptible	1,000	393 (39.3)	1.0	1.0
Monoresistance	90	19 (21.1)	**0.4 (0.2–0.7)**	0.6 (0.4–1.1)
Polyresistance	25	5 (20.0)	0.4 (0.1–1.04)	0.7 (0.3–2.1)
Multidrug resistance	6	1 (16.7)	0.3 (0.04–2.7)	0.5 (0.05–5.0)

*__Boldface__ indicates significance (p<0.05). OR, odds ratio.
†Multivariate analysis was based on purposeful selection with variables with p<0.2 in unadjusted analyses being included in the multivariate model. For Beijing strains, multivariate analysis included the independent variables of age at diagnosis and chest radiography. Sex, age at diagnosis, population group, and drug resistance were the independent variables in the multivariate analysis for non-Beijing strains.

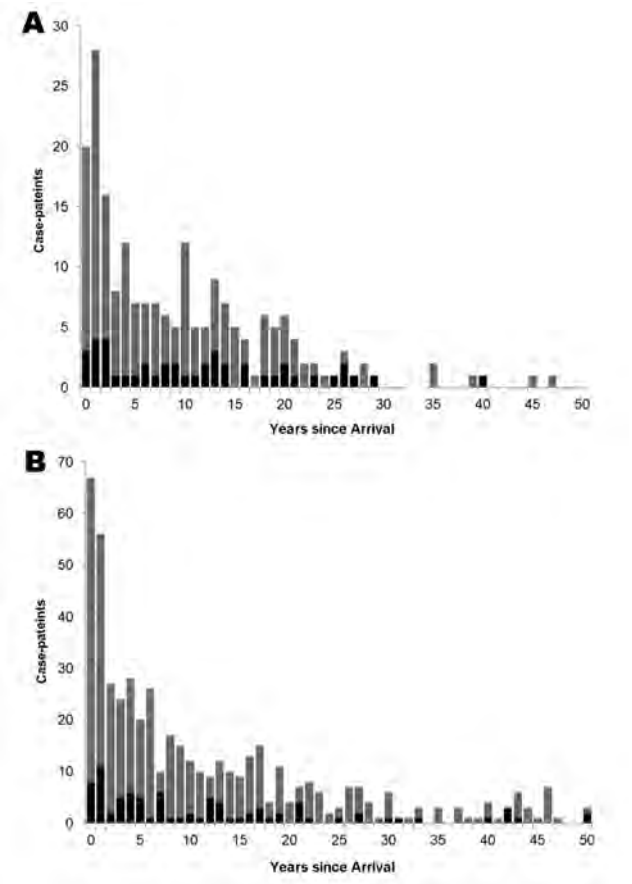

Figure. Number of clustered and nonclustered cases according to *Mycobacterium tuberculosis* lineage among foreign-born persons and time since arrival in Alberta, Canada, 1991–2007. A) Beijing cases; B) Non-Beijing cases. Gray bars, nonclustered cases; black bars, clustered cases.

number of secondary cases was associated with sputum smear–positive index case-patients (Table 3). The lineage of *M. tuberculosis* was not independently associated with the number of secondary cases.

In subgroup analyses of foreign-born index case-patients, the number of secondary cases per index case-patient was unrelated to the length of residency in Canada. For example, compared with index case-patients who were ≤2 years since arrival, the relative transmission indices of those 3–5 years and those >20 years since arrival were 1.1 (95% CI 0.3–4.1) and 1.2 (95% CI 0.4–3.6), respectively. Among persons born in the Western Pacific, no risk factors for the number of secondary cases within 2 years per index case-patient were identified, including age, *M. tuberculosis* lineage, or time since arrival (Table 4).

Sensitivity analyses demonstrated that longer cutoff periods produced higher transmission indices among Beijing cases and non-Beijing cases (Table 5). Although Beijing index cases had significantly fewer secondary cases than did non-Beijing index cases regardless of the length of the cutoff period, *M. tuberculosis* lineage was not associated with the number of secondary cases within a 2-, 3-, or 5-year cutoff period after we controlled for demographic characteristics and infectiousness. Beijing index cases had significantly fewer secondary cases than did non-Beijing cases independent of other factors when a cutoff period was not defined (equivalent to overall transmission). Inclusion of nonpulmonary secondary cases also increased the transmission indices of Beijing and non-Beijing strains but had no significant effect on relative transmission indices.

Discussion

Outbreaks of *M. tuberculosis* Beijing lineage strains in high and low TB incidence settings have had major public health implications (*7*,*24*). Notwithstanding the effect of these outbreaks, we found the transmission of Beijing strains to be similar to that of non-Beijing strains in Alberta, a low TB incidence immigrant-receiving province of Canada. Speculation about the increased transmissibility of Beijing strains also has been refuted in other low incidence immigrant-receiving countries and in The Gambia (*8*,*25*). In South Africa, findings about the transmissibility of Beijing strains have been conflicting (*2*,*26*). The general absence of evidence to suggest that Beijing strains are inherently more transmissible than other *M. tuberculosis* lineages is highly informative for TB prevention and care programs, given the propensity for multidrug-resistant TB among persons infected with Beijing strains (*6*–*8*).

M. tuberculosis is transmitted most frequently when persons with TB have positive sputum acid-fast bacilli results, especially positive results with higher semiquantitative grades, and lung cavitation (*27*,*28*). Consequently, previous findings that Beijing strains are not typically associated with sputum smear positive or cavitary disease (*6*,*29*) accords with reported similarities in the transmission of Beijing and non-Beijing strains. In our study, the infectiousness of Beijing and non-Beijing cases was similar in terms of sputum smear positivity and bacillary load, whereas the likelihood of cavitation was significantly less for Beijing cases.

That Beijing strains have been associated with increased transmission in some settings may reflect geographic variations in virulence phenotypes. In the *M. tuberculosis* complex, evolutionarily modern lineages (including the Beijing lineage) induce weaker immune responses than do ancient lineages, and this response possibly provides modern lineages with a selective advantage in terms of more rapid disease progression and transmission in the human population (*30*). An array of virulence phenotypes also have been demonstrated in the more evolutionarily recent subfamily of Beijing strains (i.e.,

the modern subfamily as characterized by the insertion of IS*6110* in the noise transfer function chromosomal region [*31*]), including differences in the pathogenic characteristics (and potential transmissibility) of closely related strains in the same sublineage (*12*,*13*). For example, strains in the modern Beijing subfamily have significant variations in their intracellular growth rates and hence significant differences in tumor necrosis factor-α levels (*13*). This variation may be of particular relevance because of higher tumor necrosis factor-α levels in the bronchoalveolar lavage fluid of TB patients with large cavities (*32*).

To better understand the potential implications of virulence phenotypes, it would be of benefit if future population-based investigations in high and low incidence settings discerned between the disease characteristics and transmissibility of different *M. tuberculosis* subfamilies or sublineages. A post hoc analysis of the IS*6110* RFLP profiles of Beijing strains in this study found that 6 (2.3%) were ≤70% homologous to the profiles of the 19 Beijing reference strains (*33*) and may therefore represent atypical/ancient Beijing strains (*31*); these 6 isolates had nonclustered IS*6110* RFLP profiles.

In agreement with previous studies (*15*,*18*,*27*), the transmission of *M. tuberculosis* in our study was lower for older and for foreign-born persons and was unrelated to drug resistance. A deeper exploration into these transmission factors in the current study also demonstrates that these factors are independent of *M. tuberculosis* lineage, at least within the broad categories of Beijing and non-Beijing lineage strains.

TB incidence among foreign-born persons in immigrant-receiving countries has a characteristic and inverse relationship with increased time since arrival (*34*). Our findings demonstrate that this characteristic relationship is clearly evident for nonclustered cases that presumably result from the reactivation of latent TB infections acquired before immigration. Clustered Beijing and non-Beijing cases also appear to follow a similar pattern. Despite the occurrence of nearly one quarter of clustered Beijing and non-Beijing cases within the first 2 years after arrival, transmission was not associated with time since arrival, a finding that concurs with a previous study (*35*). Nevertheless, time since arrival may still have major implications for the interpopulation transmission of *M. tuberculosis* (*35*). Collectively, these findings

Table 3. Risk factors for the recent transmission of *Mycobacterium tuberculosis*, Alberta, Canada, 1993–2007

Characteristic	Index case-patient		Secondary case-patient	Transmission index*	Relative transmission index (95% CI)†	
	Nonclustered	Clustered			Unadjusted	Adjusted
Sex						
F	330	29	38	0.11	1.0	
M	440	44	68	0.14	1.3 (0.9–2.0)	
Age at diagnosis, y						
<35	185	17	30	0.15	1.0	1.0
35–64	277	35	47	0.15	1.0 (0.6–1.6)	0.6 (0.4–1.0)‡
>64	308	21	29	0.09	**0.6 (0.4–1.0)**§	**0.4 (0.3–0.7)**
Population group						
Canadian-born	210	46	77	0.30	1.0	1.0
Foreign-born, other	243	9	9	0.04	**0.1 (0.1–0.2)**	**0.1 (0.1–0.2)**
Foreign-born, Western Pacific	317	18	20	0.06	**0.2 (0.1–0.3)**	**0.2 (0.1–0.3)**
Sputum smear status§						
Negative	364	25	36	0.09	1.0	1.0
Positive	357	46	68	0.17	**1.8 (1.2–2.7)**	**1.6 (1.0–2.3)¶**
Bacillary load#						
Low	134	15	25	0.17	1.0	
High	56	15	23	0.32	**1.9 (1.1–3.4)**	
Chest radiography						
No cavitation	596	44	65	0.10	1.0	
Cavitation	174	29	41	0.20	**2.0 (1.3–2.9)**	
Drug resistance						
Pan-susceptible	660	66	98	0.13	1.0	
Monoresistance	72	4	4	0.05	0.4 (0.1–1.1)	
Polyresistance	28	3	4	0.13	1.0 (0.4–2.6)	
Multidrug resistance**	10	0	0	0.00		
M. tuberculosis strain						
Non-Beijing	597	61	94	0.14	1.0	0.8 (0.4–1.7)
Beijing	173	12	12	0.06	**0.5 (0.2–0.8)**	
Total	770	73	106	0.13		

*The number of secondary cases divided by the number of index cases
†Bivariate and multivariate Poisson regression models used an offset of 1 each index case. Variables with p<0.20 in bivariate analysis were eligible for inclusion in the multivariate model. **Boldface** indicates significance (p<0.05).
‡p = 0.058.
§Sputum smear microscopy was not completed for all cases.
¶p = 0.037.
#Bacillary load was not included in multivariate modeling because of multicollinearity with sputum smear status.
**Multidrug-resistant TB was excluded from bivariate and multivariate analyses.

Table 4. Risk factors for the recent transmission of *Mycobacterium tuberculosis* among index case-patients born in Alberta, Canada, in the Western Pacific, 1993–2007

Characteristic	Index case-patients		Secondary case-patients	Transmission index*	Relative transmission index (95% CI)†	
	Nonclustered	Clustered			Unadjusted	Adjusted
Sex						
F	122	9	10	0.08	1.0	
M	195	9	10	0.05	0.6 (0.3–1.5)	
Age at diagnosis, y						
<35	83	5	5	0.06	1.0	
35–64	110	4	4	0.04	0.6 (0.2–2.3)	
>64	124	9	11	0.08	1.5 (0.5–4.2)	
Sputum smear status‡						
Negative	174	7	7	0.04	1.0	1.0
Positive	126	9	11	0.08	2.1 (0.8–5.4)	2.1 (0.8–5.5)
Bacillary load§						
Low	53	3	5	0.09	1.0	
High	17	1	1	0.06	0.6 (0.1–5.3)	
Chest radiography						
No cavitation	252	14	16	0.06	1.0	
Cavitation	65	4	4	0.06	1.0 (0.3–2.9)	
Drug resistance						
Pan-susceptible	239	13	14	0.06	1.0	
Monoresistance	51	2	2	0.04	0.7 (0.2–3.0)	
Polyresistance	21	3	4	0.17	3.0 (1.0–9.1)	
Multidrug resistance¶	6	0	0	0		
M. tuberculosis lineage						
Non-Beijing	171	9	11	0.06	1.0	1.0
Beijing	146	9	9	0.06	1.0 (0.4–2.3)	0.9 (0.3–2.2)
Time since arrival in Canada, y						
≤2	76	3	3	0.04	1.0	
3–5	43	1	1	0.02	0.6 (0.1–5.8)	
6–10	53	6	6	0.10	2.7 (0.7–10.7)	
>10	112	8	10	0.08	2.2 (0.6–8.0)	
Total	317	18	20	0.06		

*The number of secondary cases divided by the number of index cases.
†Bivariate Poisson regression using an offset of 1 each index case.
‡Sputum smear microscopy was not completed with all cases.
§Bacillary load was not included in multivariate modeling because of multicollinearity with sputum smear status.
¶Multidrug-resistant tuberculosis was excluded from bivariate analyses.

emphasize the need for screening and prevention activities in foreign-born persons as a critical means of reducing the reactivation of latent TB infection as early after arrival as possible (*36*). It also reinforces the need for high-income countries to increase their funding of efforts to expand TB care in high incidence countries (*37*).

This study reaffirms that foreign-born persons are not a major source of *M. tuberculosis* transmission (including Beijing strains) despite their high case rates (*15*,*18*,*21*). Rather, the proportion of nonclustered cases suggests that the reactivation of latent TB infection accounts for 82% of foreign-born case-patients (i.e., 80% and 83% of foreign-born Beijing and non-Beijing case-patients, respectively). The inevitable importation of pathogens, such as Beijing strains, therefore should not be viewed so much as a threat as a challenge. The challenge lies in the host country's resolve to prevent the reactivation of latent TB infection in recently arrived immigrants and in a larger population of aging immigrants while contending with constantly evolving immigration patterns (*34*).

The maintenance of a comprehensive provincial TB dataset derived through the amalgamation of TB Registry and mycobacteriology data was crucial for this study and the general evaluation of TB prevention and care in Alberta. The accuracy of strain classification also was enhanced through use of an unambiguous and validated genotyping method (*19*,*20*). Because Alberta is 1 of 4 primary immigrant-receiving provinces in Canada that has a similar immigration pattern as 2 of the other 3 (i.e., Ontario and British Columbia), the study results are anticipated to have national relevance. The generalizability of the study results to other low TB incidence immigrant-receiving countries will be influenced by the degree to which their immigration patterns are similar.

Unavoidable sampling limitations will have produced underestimates in clustering (*38*) and affected the transmission indices (*39*). Nevertheless, sampling bias was minimized in several ways: use of the provincial TB Registry for case identification; culture confirmation of >85% of TB cases in Alberta; availability of an expansive study period; and inclusion of 99% of eligible culture-confirmed pulmonary TB cases. Although a common practice in transmission studies, excluding nonpulmonary secondary cases could produce underestimates in clustering and transmission indices.

Table 5. Sensitivity analyses of the relative transmission index of *Mycobacterium tuberculosis* lineage, Alberta, Canada, 1991–2007

Variable	Transmission Index		Relative transmission index (95% CI)*	
	Beijing	Non-Beijing	Unadjusted	Adjusted†
Cutoff period, y				
2	0.06	0.14	**0.45 (0.25–0.83)**	0.82 (0.41–1.67)
3	0.09	0.18	**0.50 (0.29–0.89)**	1.04 (0.53–2.06)
5	0.11	0.33	**0.33 (0.17–0.63)**	0.86 (0.41–1.79)
Overall	0.15	0.39	**0.38 (0.26–0.54)**	**0.58 (0.39–0.87)**
Inclusion of nonpulmonary secondary cases‡	0.07	0.17	**0.42 (0.23–0.74)**	0.70 (0.36–1.36)

*__Boldface__ indicates significance (p<0.05).
†Adjusted for sex, age at diagnosis, population group, sputum smear status, lung cavitation and *M. tuberculosis* lineage.
‡Using a 2-year cutoff period.

However, sensitivity analyses in this study found the effect of this limitation to be minimal, the overall associations with transmission being unaffected by the inclusion of nonpulmonary secondary cases.

The transmission index used in this study, while advantageous for quantifying recent transmission within an expansive study period (*21*), is subject to the same limitations as other TB transmission indices (*39*). Overestimates in clustering may have resulted from the common molecular epidemiologic assumption that cases with identical DNA fingerprints were part of a transmission cluster (*4*,*27*). Although bias may have been introduced by excluding 31% of cases from the analysis of recent transmission, the effect on the study results probably is minimal because of the similarities of included and excluded cases. Last, the relatively small number of secondary Beijing cases and Beijing cases among Canadian-born persons in this study limited the ability to comprehensively assess the cross-population transmission of Beijing strains and the strain-specific transmission patterns in Canadian-born Aboriginal peoples.

This study demonstrated that Beijing strains are not independently associated with increased clustering or a larger number of secondary cases than non-Beijing strains in a setting with comprehensive and effective TB prevention and care practices (*40*). Combined with the uncommon transmission of *M. tuberculosis* by foreign-born persons in this and other studies that led to disease (*15*,*18*), there appears to be little cause for concern about the importation and subsequent transmission of Beijing strains in low TB incidence immigrant-receiving settings.

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Foodborne Transmission of Bovine Spongiform Encephalopathy to Nonhuman Primates

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Risk for human exposure to bovine spongiform encephalopathy (BSE)–inducing agent was estimated in a nonhuman primate model. To determine attack rates, incubation times, and molecular signatures, we orally exposed 18 macaques to 1 high dose of brain material from cattle with BSE. Several macaques were euthanized at regular intervals starting at 1 year postinoculation, and others were observed until clinical signs developed. Among those who received ≥5 g BSE-inducing agent, attack rates were 100% and prions could be detected in peripheral tissues from 1 year postinoculation onward. The overall median incubation time was 4.6 years (3.7–5.3). However, for 3 macaques orally exposed on multiple occasions, incubation periods were at least 7–10 years. Before clinical signs were noted, we detected a non-type 2B signature, indicating the existence of atypical prion protein during the incubation period. This finding could affect diagnosis of variant Creutzfeldt-Jakob disease in humans and might be relevant for retrospective studies of positive tonsillectomy or appendectomy specimens because time of infection is unknown.

Variant Creutzfeldt-Jakob disease (vCJD) (*1*) is most likely caused by dietary exposure to bovine spongiform encephalopathy (BSE) prions (*2–4*). In the United Kingdom, risk for infection with BSE has been considerable, but only 172 cases of vCJD have been documented (*5–7*). However, the infective dose for oral transmission of the BSE agent to humans is unknown, and incubation times can only be estimated (*5*,*6*). In 2001, the European Union funded a risk assessment study in nonhuman primates to estimate the risk for humans exposed to BSE-contaminated food or blood products (*8*).

A determining factor for susceptibility to BSE prions is a polymorphism for methionine (M) or valine (V) at codon 129 of the human prion protein gene (*PRNP*). All vCJD cases examined were methionine homozygotes at *PRNP* codon 129 (129-MM) (*9*). The overall distribution of *PRNP* codon 129 genotypes in the general UK population is ≈39% MM, ≈50% MV, and ≈11% VV (*7*,*10*). Evidently, persons with a 129-VV genotype can be infected (*11*), and clinical signs develop after a longer incubation time among those with a 129-MV genotype than among those with a 129-MM genotype (*12*). However, retrospective analyses of biopsy samples suggest that prevalence of BSE infection is higher among persons who belong to a certain birth cohort and lived in the United Kingdom from 1980 through 1989 (109 cases/million persons [*13*] to 237 cases/million persons [*14*]). The reason for the discrepancy between the low number of vCJD cases and higher prevalence of infected persons in the United Kingdom is not known, but the *PRNP* polymorphism might contribute to this discrepancy as just described. Intriguingly, among hamsters that were orally exposed multiple times to central nervous system (CNS) tissues infected with the scrapie agent, incubation times were significantly prolonged (*13*). Thus, not only the *PRNP* polymorphism and the dose but also the mode of transmission might contribute to the development of subclinical cases. However, estimating exposure risks for humans based solely on these results is difficult because of the digestive physiology, life expectancy, and other metabolic parameters of hamsters.

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In prion diseases such as CJD, kuru, BSE, scrapie, and chronic wasting disease, the cellular form of prion protein (PrP^{C}) is thought to be converted into abnormal PrP (PrP^{Sc}) through a posttranslational event. As a result, PrP^{Sc} becomes partially resistant to proteases. The misfolded prion protein comprises an N terminal protease-sensitive part followed by a region with variable protease sensitivity and a C-terminal protease-resistant core referred to as PrP^{res} or PrP27–30 (Figure 1). Limited protease exposure of PrP^{Sc} in vitro generates nonglycosylated core fragments of 19–21 kDa (*14*,*15*), which are used to distinguish 2 major PrP^{Sc} types by electrophoresis. Type 1 core protein has an apparent molecular mass of 21 kDa. Its primary cleavage site is at residue 82. Type 2 core protein migrates to the 19-kDa region and has a primary cleavage site at residue 97 (*16*,*17*). Both types can coexist in a considerable number of sporadic CJD (*18*,*19*) and vCJD cases (*14*). Subtypes and strains can be further characterized by their so-called glycoform profile because the nonobligatory addition of 1–2 sugar chains results in 3 differently glycosylated isoforms in PrP^{C} and PrP^{Sc} (non-, mono-, and diglycosylated molecules, referred to as a PrP^{res} triplet). In sporadic CJD type 2, the monoglycosylated isotype predominates and is referred to as a type 2A signature; whereas, in vCJD, the diglycosylated isoform predominates (*1*) and is referred to as a type 2B signature (*14*,*16*,*19*). Additional PrP^{res} fragments have been described, for example the so-called C-terminal fragments of 12/13 kDa (*20*) and 17 kD (*21*).

Results of the European Union–funded nonhuman primate risk assessment study, designed to determine the dose at which 50% of macaques will be infected (*8*), show that a 5-g dose given on 1 occasion infected all macaques. Moreover, multiple exposures to high doses might prolong incubation time. Intriguingly, a non–type 2B PrP^{res} pattern in CNS tissues of macaques during the preclinical phase indicated the existence of an intermediate prion isoform. This finding might be relevant for retrospective studies of tonsillectomy or appendectomy specimens, because the time point of infection in humans with PrP^{res}-positive biopsy specimens is not known. As part of the European Union–funded study, we aimed to determine attack rates and incubation times after oral exposure to 5 g or 16 g of BSE-infected brain material in adult cynomolgus monkeys (*Macaca fascicularis*).

Methods

We orally exposed 18 macaques, each 5 years of age, to brain material from cattle with BSE: 15 macaques on 1 occasion and 3 macaques on multiple occasions. Most animals were kept at the primate center of the Paul-Ehrlich-Institut under biosafety level 3 conditions; the others (macaques M3–M8) were kept at the Swedish Institute for Infectious Disease Control, Stockholm. The study was approved by the Hessian Animal Protection Committee (local permit no. F107/45 and F107/63) and supervised by local authorities (Regierungspräsidium Darmstadt).

The BSE inoculum was a pool of homogenized bovine brain from 11 cows with natural BSE infection (*22*). The level of infectivity was determined (data not shown) in BoTg110 mice (*23*). Cynomolgus monkeys were purchased from the Centre de Recherche en Primatologie, Mauritius. All animals were homozygous for M at codon 129 of the *PRNP* gene (*4*,*22*). We fed (in muesli balls, monitored by video to ensure that the total amount was eaten) 5 g of BSE inoculum to each of 12 macaques (macaques S1–S5 and S9–S15), 8–16 g of BSE inoculum to each of 6 macaques (macaques S6–S8 and C1–C3), and mock inoculum (non-BSE–infected brain material) to each of 8 macaques (macaques M1–M8). We had originally planned to inoculate all macaques on 1 occasion; however, because of feeding problems, 3 macaques (C1–C3) had to be inoculated on several occasions. Of these 3 macaques, 1 received a cumulative dose of 8 g, the second 10 g, and the third 16 g (Table 1).

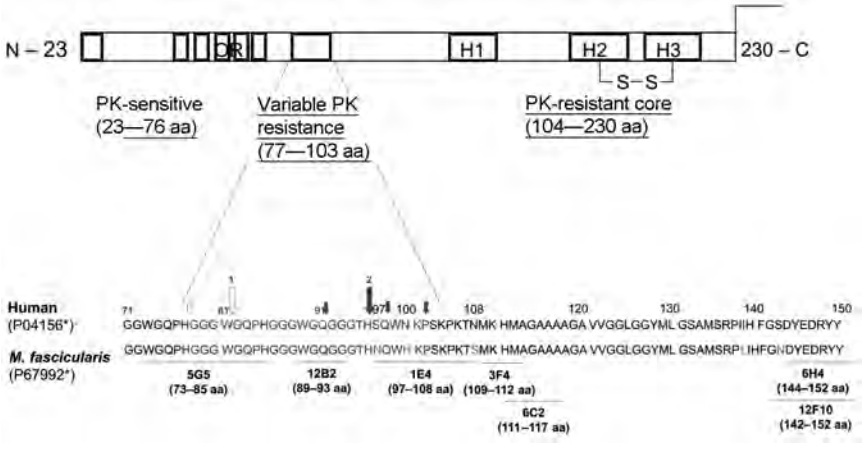

Figure 1. Schematic diagram of the mature nonglycosylated prion protein and below amino acid sequences of the human and the simian prion polypeptide chain. Homology (198/207 aa) between human and simian mature cellular form of prion protein on the amino acid level is 96%. Large and small arrows indicate major and minor, respectively, proteinase K digestion sites (*14*). Open arrows indicate digestion sites in type 1 fragments; filled arrows indicate digestion sites in type 2 fragments. The epitopes recognized by the used monoclonal antibodies are shown below. OR, octarepeat region; H, α-helical structure; PK, proteinase K; *M. fascicularis, Macaca fascicularis.* *Swiss-Prot (www.ebi.ac.uk/swissprot/) accession numbers.

Table 1. Schedule for successive oral inoculation of 3 macaques with brain tissue from BSE-infected cattle*

Macaque	Dose, g				
	Day 1	Day 2	Day 3	Day 7	Cumulative
C1	5.0	2.0	None	1.0	8.0
C2	6.4	2.6	None	1.0	10.0
C3	9.6	3.5	2.9	None	16.0

*BSE, bovine spongiform encephalopathy.

All animals were observed daily for any abnormalities. Cerebrospinal fluid (CSF) samples were collected at regular intervals and examined for biomarkers of brain damage by 14–3-3 protein (14–3-3p) tests (*22*). We planned 2 studies. For study 1, BSE-infected macaques (groups I and III) were to be kept until development of clinical signs to determine incubation periods. For study 2, macaques (group II) received 5 g BSE brain material on 1 occasion and were euthanized at regular intervals during the incubation period (macaques S9–S15, Table 2). However, study 1 was possible only for macaques S1–S8 (group I, Table 2) because among macaques C1–C3 (group III, Table 2), non–BSE-associated disease necessitated euthanasia. Macaques M1–M8 were the control macaques (group IV) inoculated with non-BSE brain material (Table 2).

During postmortem examinations, brain, spinal cord, gut-associated lymphoid tissues (GALT), lymph nodes, tonsils, and other organs and tissues were either fixed in buffered formaldehyde solution (4% wt/vol) or stored at −80°C as described (*22*). Routine histopathologic examinations of the brains were performed to detect spongiform lesions in hematoxylin and eosin–stained tissue sections. To detect proteinase K (PK)–resistant PrP fragments in tissue sections, we conducted paraffin-embedded tissue blot analyses (*24*). Western immunoblot analyses to localize PrP^{res} in homogenized and PK–treated (20 μg PK/mL buffer) tissue samples (50 μg of tissue proteins were loaded onto a lane) were conducted as described (*22*). Western blot–negative results were retested by using Amersham Hyperfilm ECL (GE Healthcare, Life Sciences Europe, Freiburg, Germany) for visualization. Monoclonal antibodies and polyclonal antiserum were used

Table 2. Characteristics of oral inoculation of 5-year-old macaques with BSE-inducing or mock brain material *

Macaque	Sex	BSE dose, g	Euthanized, y postinoculation	14-3-3p–positive CSF	PrP^{res} pattern (brain)	PrP^{res} pattern (spinal cord C_1–T_{12})	PrP^{res} pattern (spinal cord L_1–L_4)
Group I (clinically infected)†							
S1	F	5	4.3	Yes	2B	2B	2B
S2	F	5	4.6	Yes	2B	2B	2B
S3	M	5	4.7	Yes	2B	2B	2B
S4	F	5	4.8	Yes	2B	2B	2B
S5	F	5	5.2	Yes	2B	2B	2B
S6‡	F	16	3.7	Yes	2B	2B	2B
S7	F	16	4.5	Yes	2B	2B	2B
S8	F	16	5.3	Yes	2B	2B	2B
Group II (preclinical)§							
S9	M	5	1.0	No	Neg	Neg	Neg
S10	M	5	1.0	No	Neg	Neg	Neg
S11	M	5	3.0	No	Neg	Neg	Non-2B
S12	M	5	3.0	No	Neg	Neg	Non-2B
S13	M	5	3,9	No	Neg	$Th_{7–10}$+	Neg
S14	F	5	4,1	No	Neg	Neg	Non-2B
S15	F	5	5.0	No	Neg	Neg	Non-2B
Group III (preclinical)¶							
C1	F	8	6.5	No	Neg	Neg	Non-2B
C2	F	10	6.5	No	Neg	Neg	Non-2B
C3	F	16	8.8	No	Neg	Neg	Neg
Group IV (controls)#							
M1	F	5	2.0	No	Neg	Neg	Neg
M2	F	5	5.0	No	Neg	Neg	Neg
M3	F	16	6.0	No	Neg	Neg	Neg
M4	F	16	6.0	No	Neg	Neg	Neg
M5	F	16	6.0	No	Neg	Neg	Neg
M6	F	16	6.0	No	Neg	Neg	Neg
M7	F	0.05	6.0	No	Neg	Neg	Neg
M8	F	0.05	6.0	No	Neg	Neg	Neg

*BSE, bovine spongiform encephalopathy; PrP^{res}, proteinase-resistant prion protein; CSF, cerebrospinal fluid; C, cervical; T, thoracic; L, lumbar; neg, negative.
†Received 1 BSE dose, observed until onset of clinical signs.
‡Macaque S6 had a highly stimulated immune system on the day of oral exposure and thereafter (reason unknown) and had the highest postmortem levels of proteinease-resistant prion protein (PrP^{res}) in spleen and other lymphoreticular tissues of all examined macaques (data not shown).
§Animals received 1 BSE dose and were euthanized at regular intervals during incubation period (all animals had PrP^{res} -positive non–central nervous system tissue).
¶Cumulative BSE dose, euthanized.
#Exposed to mock (non–BSE-infected) bovine brain material and euthanized during aging to act as age-matched controls.

Table 3. Products used for Western immunoblot and paraffin-embedded tissue blot analyses*

mAb or antiserum	Linear epitope (amino acid) on human PrP	Source (reference)
mAb 8B4	37–44	Santa Cruz Biotechnology, Santa Cruz, CA, USA
mAb 5G5	73–85	(*25*)
mAb 12B2	89 –93	CIDC, Lelystad, the Netherlands (*14*)
mAb 1E4	97–108	Sanquin BV, Amsterdam, the Netherlands (*14*)
mAb 3F4	109–112	Abcam, Cambridge, UK
mAb F89-160-1-5	142–152	Calbiochem/Merck4Biosciences, Darmstadt, Germany
mAb 1C5	119–130	(*26*)
mAb 6H4	144–152	Prionics AG, Schlieren, Switzerland
mAb 12F10	142–152	SPI-Bio/IBL Int., Hamburg, Germany
C-20 antiserum	220–231	Santa Cruz Biotechnology, Santa Cruz, CA, USA
mAb BAR 236	213– 251	SPI-Bio/IBL Int., Hamburg, Germany

*mAb, monoclonal antibody; PrP, prion protein.

for immunodetection (Table 3) and epitope mapping of PrP^{C} and PrP^{Sc} (Figure 1). Bioassay studies were conducted in BoTg110 mice expressing the bovine *PRNP* gene (*23*).

Results

Among single-dosed macaques, gait ataxia developed in S1–S8, and CSF samples were positive for 14–3-3p from 3.7 through 5.3 years postinoculation; no differences in incubation periods were noted for macaques that received 5 g or 16 g of BSE inoculum (Table 2, Figure 2). Postmortem examinations of all macaques showing neurologic signs detected a type 2B PrP^{res} signature in different brain areas (obex region, cerebellum/deep nuclei, pons, thalamus, caudate nucleus, cortex cerebri) and in all spinal cord segments examined (C_1–L_4) (Table 2, Figures 3, 4). The type 1–specific monoclonal antibody 12B2 could not detect any PrP^{res} triplets in these specimens (Figure 5, panel A). Typical spongiform changes were seen in hemotoxylin and eosin–stained brain tissue sections (data not shown).

Among macaques that received 8–16 g of BSE inoculum (C1–C3), no behavioral changes, gait ataxia, or 14–3-3p-positive CSF were detected. However, as they got older, obesity and chronically elevated blood glucose concentrations (>126 mg/dL) developed and were followed by a rapid decrease in body weight. For humane reasons, these animals were euthanized at 6.5 years (C1, C2) and 8.8 years (C3) postinoculation. Retrospective analyses of plasma samples detected normal insulin levels at 3–4 years of age, followed by hyperinsulinemia and a progressive decline in plasma insulin levels. Postmortem examinations of pancreatic tissue sections indicated replacement of the normal islet architecture by islet-associated amyloid and marked reduction of α- and β-cell mass (data not shown). On the basis of the typical changes during the prediabetic phase, clinical parameters, and pancreas histopathology, type 2 diabetes (a common disease for nonhuman primates in captivity) was diagnosed. We detected neither spongiform changes nor PrP^{res} deposits in the brains of these 3 macaques (Table 2). However, an atypical PrP^{res} pattern, a 26-kDa fragment, and a 17-kDa fragment were found in lumbar, but not other, spinal cord segments in 2 of the 3 macaques (Table 2, Figure 4). Paraffin-embedded tissue blot analyses confirmed Western immunoblot results (Figure 6). Paraffin-embedded tissue blot analyses of lymphoid tissues (GALT, lymph nodes) revealed PrP^{res} deposits in 3 macaques (Table 2).

From 3 years postinoculation onward, we also detected an atypical PrP^{res} pattern in lumbar spinal cord segments in 4 of 6 macaques that had received a single 5-g dose of BSE inoculum (macaques S11, S12, S14, S15; Table 2, Figure 4). The lumbar part of the spinal cord is probably the primary site of prion entry into the simian CNS after oral uptake of the BSE agent (data not shown). We did not detect histopathologic changes in hemotoxylin and eosin–stained brain tissue sections from macaques before clinical signs developed (S9–S15, C1–C3).

The atypical molecular signature found in lumbar segments of macaques with subclinical infection was characterized by the predominance of a PrP^{res} fragment,

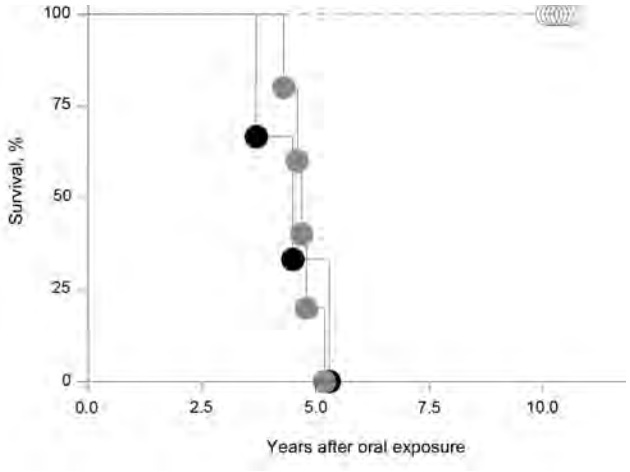

Figure 2.Percentage macaques surviving after oral inoculation brain material with or without (mock) bovine spongiform encephalopathy (BSE)–inducing agent. Macaques exposed to 5 g (gray circles) or 16 g BSE (black circles) on 1 occasion and mock controls (open circles) are shown. The median incubation times for those given 16 g and 5 g BSE each was 4.7 years and 4.6 years, respectively. The difference was statistically not significant.

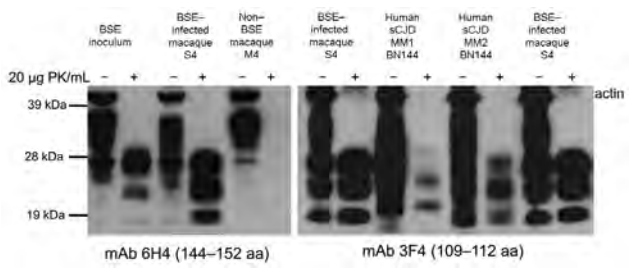

Figure 3. Western immunoblot analysis of proteinease-resistant prion protein (PrPres) (from brain). The size of the nonglycosylated band was either 21 kDa (termed type 1, sCJD case BN141) or 19 kDa (termed type 2, sCJD case BN144). Diglycosylated PrPres predominated in the BSE inoculum and in all macaques showing neurologic signs (macaque S4 is shown as a representative example for all symptomatic cases), and the pattern is termed type 2B to distinguish it from type 2 cases in which the monoglycosylated form predominated (Type 2A, sCJD case BN144). Two different mAbs were used for routine immunodetection: 6H4 (left panel) and 3F4 (right panel). Blots were co-stained with a polyclonal anti-actin antiserum. BSE, bovine spongiform encephalopathy; sCJD, sporadic Creutzfeldt-Jakob disease; PK, proteinase K; mAb, monoclonal antibody.

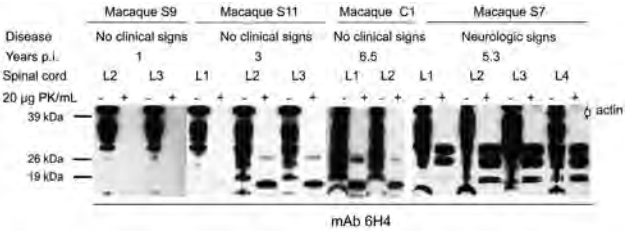

Figure 4. Western blot analysis of bovine spongiform encephalopathy proteinease-resistant prion protein (PrP^{res}) (lumbar spinal cord segments) in preclinical and clinically ill macaques. An atypical PrP^{res} pattern was detectable in macaques euthanized during incubation from 3 years p.i. (macaque S11) to 6.5 years p.i. (macaque C1). All samples were co-stained with an anti-actin-antiserum. p.i., postinoculation; PK, proteinase K; mAb, monoclonal antibody.

which migrated at the 17-kDa region of the sodium dodecyl sulfate polyacrylamide gel. A second PrP^{res} molecule migrated at the area of 26 kDa but was sometimes hardly visible (Figure 4). Epitope mapping was conducted to characterize these 2 PrP molecules. Paradoxically, all antibodies, including monoclonal antibody 12B2 (Figure 5, panel B), and polyclonal antiserum recognizing the C-terminus (data not shown) could bind to both fragments. Non-PrP antibodies did not bind to the atypical fragments (data not shown). Deglycosylation of PK–treated lumbar spinal cord samples by using peptide N-glycosidase F treatment resulted in a single band with a molecular weight of ≈22–23 kDa in animals with preclinical/subclinical infection compared with a single 19–20 kDa band in macaques showing neurologic signs (data not shown).

The reason for the abnormal migration behavior of the atypical fragments remains to be determined. When lumbar spinal cord tissue homogenates were intracerebrally inoculated into mice transgenic for the bovine *PRNP* gene, samples from symptomatic (type 2B signature) and asymptomatic macaques (abnormal signature) were infectious and caused disease in mice (53% diseased mice in both groups) with no statistically significant difference in the incubation periods (355 ± 41 vs. 372 ± 7 days postinoculation, respectively, P_{logrank} test not significant) (Figure 7). The percentage of mice inoculated with type 2B or non–type 2B material that showed clinical signs was low, probably because of the small amount of gray matter (PrP^{res}) in the inoculum; attack rates were 100% among mice inoculated with gray matter from brain samples (data not shown). Among mice infected with tissue samples from the asymptomatic animals, a molecular signature that differed from that of the inoculum developed, whereas the type 2B signature found in macaques showing neurologic signs was stable after transmission to mice (Figure 7).

Discussion

Because only a few macaques have died, it will take longer than previously estimated before all data from low-dose (0.05 and 0.005 g) exposures will be available (data not shown). Although all macaques were originally to be inoculated with BSE-infected cattle brain material on only 1 occasion, 3 of 6 macaques receiving >5 g of inoculum had to be fed on multiple occasions.

The attack rate after dietary exposure to ≥5 g BSE brain material in 5-year-old adult 129-MM cynomolgus monkeys was 100% (18/18). PrP^{res} deposits could be detected outside the GALT in gut-draining lymph nodes from at least 1 year postinoculation onward. These data show that the dose at which 50% of these nonhuman primates will be infected will be distinctly lower than previously estimated (*4*).

For single-dosed animals, the incubation period was 4.6 years (median, range 3.7–5.3 years). There was no difference between those that received 5 g and 16 g, indicating that ≤5 g represented the dose at which disease developed in 100% of macaques (LD_{100}). The shortest incubation period was detected in macaque S6, which was given 16 g of inoculum on 1 occasion. This short incubation period might have resulted from the extremely high dose. However, retrospective analyses revealed that macaque S6 had a highly stimulated immune system (data not shown), which might also explain the short incubation period. The low variability of incubation periods (4.3–5.3 years, excluding 3.7 years for macaque S6) was probably the result of the high dose. When we inoculated macaques with lower doses (data not shown) or when macaques were inoculated on multiple occasions, incubation periods were highly variable. After the macaques received multiple oral doses, clinical signs of a spongiform disease had not

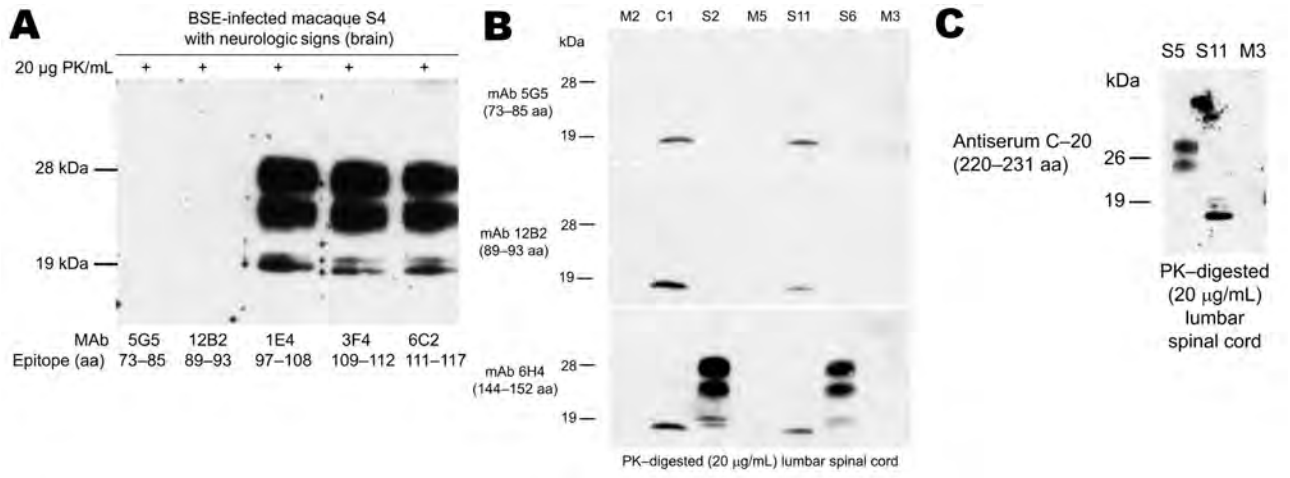

Figure 5. A) Epitope mapping of proteinease-resistant prion protein (PrPres) by Western immunblot analyses (thalamus) from a macaque showing neurologic signs. The PK-sensitive N terminal fragment (mAb 5G5) and the adjacent region showing a variable PK sensitivity (mAb 12B2) were completely digested by the proteinease. MAbs 1E4, 3F4, and 6C2 detected the PrPres triplet termed type 2B signature. B) Epitope mapping of PrPres by Western blot analyses (lumbar spinal cord segment L2) in clinically ill (S2, S6), subclinical (C1), and preclinical (S11) macaques. In preclinical macaques, mAbs 5G5, 12B2, and 6H4 detected a 17 kDa PrPres fragment. Tissue samples from mock (M) controls were completely negative for PrPres. C) Epitope mapping of PrPres by Western blot analyses (lumbar spinal cord segment L2) in a clinically ill macaque (S5), a preclinical macaque (S11), and a non–BSE-infected age-/sex-matched control macaque (M3). PK-treated tissue homogenates from preclinical macaques could also be immunostained with antiserum C20 directed against the C-terminus of the atypical 17-kDa fragment. BSE, bovine spongiform encephalopathy; PK, proteinase K; mAb, monoclonal antibody; M2, M3, M5, noninfected macaques.

developed by 6.5 (2 of 3 macaques) to 8.8 years (1 of 3 macaques) postinoculation although they received an LD_{100} on day 1 (Table 1).

Unfortunately, type 2 diabetes developed in all 3 of these macaques as they aged, and they had to be euthanized for humane reasons at the indicated time points. At postmortem examinations, lumbar spinal cord segments were PrP^{res} positive for the 2 macaques (C1 and C2) euthanized 6.5 years postinoculation. We estimate that incubation periods in these 2 animals must be at least 7 years because it took ≥6 months until PrP^{res} deposits were also detectable in the cerebellum/cortex cerebri, thereby causing clinical signs (data not shown). In the third macaque (C3) euthanized 8.8 years postinoculation with a cumulative dose of 16 g, PrP^{res} deposits could only be detected outside the CNS, thereby indicating an estimated incubation period >10 years. Similar results have been described for hamsters orally infected with the scrapie strain 263K on a single or multiple occasions. In that study, a cumulative dose significantly prolonged incubation periods, although hamsters were given much lower doses than were the macaques (*13*). The upper reference margin using 3× the standard deviation (3σ) of animal group I is 1.52 years, corresponding to a calculated upper limit of the incubation period of 6.1 years after a single high-dose exposure (5–16 g each). This calculated incubation period is distinctly lower than the estimated incubation time of ≥7–10 years within animals of group III, indicating a biological effect of the successive BSE challenge mode on the incubation time in the macaque model.

The discrepancy between the low number of vCJD cases in the United Kingdom to date and the higher prevalence

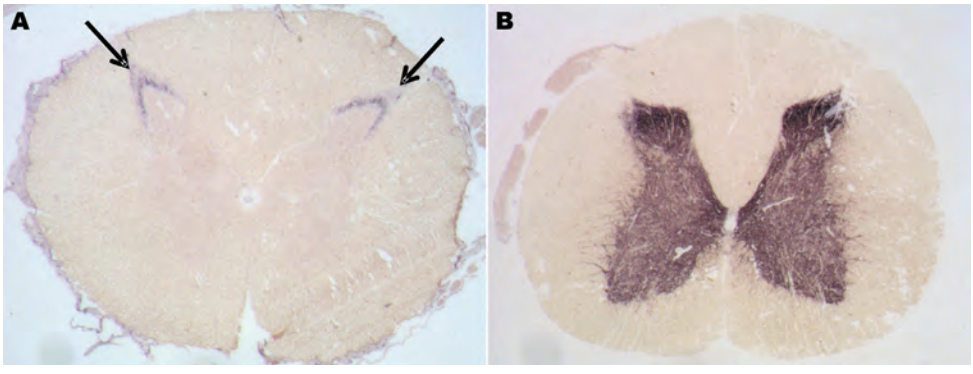

Figure 6. Paraffin-embedded tissue blot analyses of lumbar spinal cord segments from the preclinical macaque S14 (A) and a clinically ill macaque (B) for detection and localization of proteinease-resistant prion protein (PrPres) deposits. These deposits could be detected in the substantia gelatinosa (arrows) of preclinical cases.

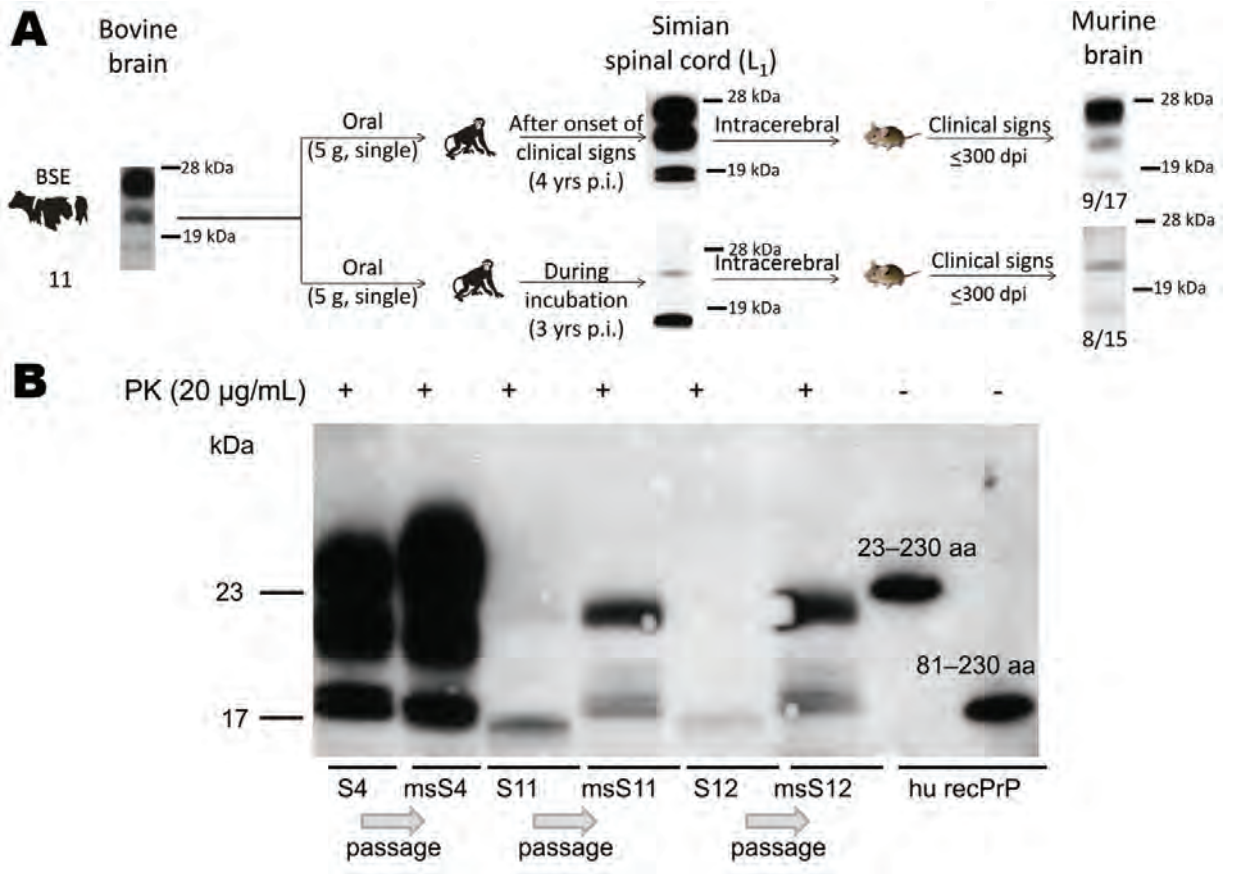

Figure 7. A) Summary of studies of BSE transmission to macaques and subsequent passage of lumbar spinal cord tissues (L_2–L_3) from a symptomatic and a preclinical macaque to BoTg110 mice. Inoculation of typical type 2B and atypical material from macaques to transgenic mice caused disease in 9 (53%) of 17 mice and in 8 (53%) of 15 mice, respectively, with no significant differences in incubation periods. B). Western immunoblots of PK-treated lumbar spinal cord samples from BSE-infected macaques (S4, S11, S12) and the corresponding bioassay results (msS4, msS11, msS12) in BoTg110 mice inoculated with simian lumbar spinal cord tissue homogenates. Analyses of the PrP^{res}, proteinase-resistant prion protein; (PrP^{res}) profile by Western immunoblot using mAb 6H4 showed a conserved BSE glycoform signature after the first passage when typical type 2B material collected from a macaque showing neurologic signs was inoculated into BoTg110 mice. However, the atypical PrP^{res} pattern detected in preclinical cases was not stable after the first passage into BoTg110 mice. Recombinant PrP fragments (23–230 aa and 81–230 aa) were included to characterize molecular weights of the atypical fragments. BSE, bovine spongiform encephalopathy; p.i., postinoculation; dpi, days postinoculation; PK, proteinase K; hu recPrP, human recombinant PrP.

of infected humans estimated on the basis of retrospective biopsy analyses (*27*,*28*) indicates the existence of pre- or subclinical cases, perhaps as a result of a low-dose exposure to BSE-contaminated material or a less susceptible *PRNP* genotype. We showed that multiple exposures to high doses of BSE prolonged incubation periods in a nonhuman primate model. These findings show that a successive BSE challenge mode might contribute to the development of pre- or subclinical cases despite a susceptible *PRNP* phenotype and an LD_{100}. This finding is relevant because it is quite likely that most of the UK population has been exposed to BSE-contaminated food on multiple occasions (*5*,*6*).

The underlying mechanism of a prolonged incubation period after multiple exposures to an agent that induces a transmissible spongiform encephalopathy is not known (*6*,*13*). Theoretically, interference between types or strains could have caused this phenomenon, as has been shown by others (*14*,*19*,*29*,*30*). However, Diringer et al. (*13*) used 1 well-defined laboratory scrapie strain (263K) that could also cause prolonged incubation periods in Syrian hamsters after multiple oral exposures. Their finding shows that at least 1 other not-yet identified mechanism causes prolonged incubation periods after multiple oral exposures to agents that induce transmissible spongiform encephalopathy.

Unexpectedly, we detected a non–type 2B PrP^{res} pattern in preclinical cases from 3 years postinoculation onward. Transmission studies in BoTg110 mice showed that tissues were infectious but that this atypical molecular signature was not stable after the first passage to transgenic mice carrying the bovine PrP gene (Figure 7). However,

the PK-sensitive N terminal part, the variable region of PK, and the C-terminal end were detectable in both atypical PrP molecules by epitope mapping studies. Thus, at least the 17-kDa molecule showed migration behavior on sodium dodecyl sulfate polyacrylamide gel electrophoresis, which did not correlate with its formal molecular weight. Posttranslational modifications can cause a gel-shifting phenomenon (i.e., anomalous gel mobility), as observed for the phosphorylated tau protein (*31*). However, it remains to be determined which mechanism caused this anomalous gel mobility. This atypical signature probably reflects neither types nor strains but rather an intermediate conformation of the pathologic PrP.

In conclusion, the LD_{100} of brain from BSE-infected cattle for 129-MM 5-year-old adult macaques exposed on 1 occasion is ≤5 g. However, this dose did not cause disease within a prolonged incubation time when animals were exposed on multiple occasions. This finding may be relevant for modeling exposure risks for foodborne prion diseases including chronic wasting disease (*32*). Moreover, the time-dependent shift of the molecular signature might be relevant for retrospective analyses of biopsy samples most likely from animals with pre- or subclinical vCJD.

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Dr Holznagel is a senior staff scientist at the Paul-Ehrlich-Institut. He performed all animal experiments and designed and supervised all laboratory work for this study.

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Populations at Risk for Alveolar Echinococcosis, France

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During 1982–2007, alveolar echinococcosis (AE) was diagnosed in 407 patients in France, a country previously known to register half of all European patients. To better define high-risk groups in France, we conducted a national registry-based study to identify areas where persons were at risk and spatial clusters of cases. We interviewed 180 AE patients about their way of life and compared responses to those of 517 controls. We found that almost all AE patients lived in 22 *départements* in eastern and central France (relative risk 78.63, 95% CI 52.84–117.02). Classification and regression tree analysis showed that the main risk factor was living in AE-endemic areas. There, most at-risk populations lived in rural settings (odds ratio [OR] 66.67, 95% CI 6.21–464.51 for farmers and OR 6.98, 95% CI 2.88–18.25 for other persons) or gardened in nonrural settings (OR 4.30, 95% CI 1.82–10.91). These findings can help sensitization campaigns focus on specific groups.

Alveolar echinococcosis (AE) is caused by the larval stage of the fox tapeworm *Echinococcus multilocularis*. In human infections, after a person ingests eggs, the metacestode cells of *E. multilocularis* proliferate in the liver, inducing a hepatic disorder mimicking liver cancer (*1*). Complete resection of liver lesions is possible in only one third of the cases, and parasitostatic and sometimes parasiticidal (*2*) treatment is available with benzimidazole compounds (albendazole or mebendazole). AE, observed only in the Northern Hemisphere, is linked to environmental features, such as land use for cattle breeding (pastures), which promotes high densities of rodents (main reservoir for the parasite) and thus a high prevalence of infection in foxes, which increases the environmental reservoir of the parasite (*3*,*4*). That the intermediate and final hosts of the parasite are members of wildlife species, largely explains why AE is an occupational disease of farmers and especially of cattle breeders (*4*). Individual risk factors vary greatly, however, depending on the country (*1*).

In Europe, the main AE-endemic areas are north of the Alps, primarily in Switzerland, France, Germany, and Austria, but recent studies showed that AE has spread during the past 20 years (*5*,*6*). Human AE cases have been diagnosed in countries previously considered free of the infection, such as Poland, Slovakia, Lithuania, Slovenia, Belgium, and Hungary (*7*,*8*). Molecular typing of *E. multilocularis* specimens collected in Europe showed that the European AE focus can be drawn as a core located in central Europe, flanked by neighboring regions where the parasite is less genetically diverse (*6*,*9*). In addition to the centrifugal spread of the disease, some epidemiologic studies also showed a significant trend of an increase in human AE incidence in some previously known foci, for example, in Switzerland (*10*). Schweiger et al. hypothesized that in Switzerland the increase in the fox population in rural and urban areas and high prevalence of *E. multilocularis* in foxes led to an increase in the infection risk for humans and the emergence of AE 10–15 years after infection increased in foxes (*10*).

France represents the western border of the European focus of AE and accounted for 235 (42%) of the 559 patients recorded in Europe during 1982–2000 (*7*). Hegglin et al. (*11*) have pointed out that AE is poorly known in France (only 88 [17.6%] of 500 interviewed persons were aware of it). This study reinforced the conclusion that better information is needed to identify at-risk populations.

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In particular, to avoid alarming the general population, we need to accurately define areas where persons are at risk for AE, identify exposed populations, and clarify behavior associated with AE contamination. Since the EurEchinoReg project (*7*), the FrancEchino Network has maintained a registry in France of AE cases, with the support of the French National Institute of Public Health Surveillance (Institut de Veille Sanitaire) (*12*). From 1982 through 2007, this registry helped identify 407 new patients in France (*13*). We present the results of a registry-based study in which we aimed to better define high-risk target groups for prevention campaigns.

Materials and Methods

FrancEchino Registry

Initiated by the EurEchinoReg Network, the FrancEchino Registry actively gathers information about AE patients and related data observed in France since 1982 as described (*12*). During 1997–1998, French AE patients were found by sending questionnaires to all hospital departments of medicine, radiology, and abdominal surgery that look after patients with AE, to all pathologists and parasitologists in France, and to all public hospital pharmacies that could deliver albendazole or mebendazole for treatment. If no response was received to 2 mailed questionnaires, telephone calls were made. Clinicians, radiologists, pathologists, pharmacists, and biologists were asked to report all suspected AE patients diagnosed since 1982, that is, patients who had a positive specific serologic test result, a compatible imaging result, characteristic histopathologic features, and had received albendazole or mebendazole treatment for >30 days. During 1998–2007, the database was updated every 2 years following the same procedure. Previously unreported patients were also identified with the support of national medical societies (French societies of infectious diseases, parasitology, gastroenterology, digestive surgery, and the French association for the study of the liver). All suspected cases were further investigated by interviewing physicians using a questionnaire that addressed the patient's AE clinical history.

Patients were classified into the following 4 groups, according to the recommendations of the World Health Organization Informal Working Group on Echinococcosis (*14*,*15*): "1) possible case—any patient with compatible clinical and epidemiologic history and imaging findings, *or* serology positive for AE; 2) probable case—any patient with clinical and epidemiologic history and imaging findings, *and* serology positive for AE with 2 tests; 3) confirmed case—the above, plus a) histopathology compatible with AE and/or b) detection of *E. multilocularis* nucleic acid sequences in tissue obtained through surgery or percutaneous biopsy; 4) all other patients were excluded from the study."

Questionnaires and Data Analysis

Patients with possible, probable, or confirmed AE were further investigated by a questionnaire addressing the epidemiologic context of their infection. Questions explored the patient's life, using each past address to search for risk factors. Six binary questions were asked about behavior regarding picking wild berries, eating raw salads, hunting, having a kitchen garden, and having contacts with dogs and foxes. Interviews were carried by telephone by a physician or during a medical consultation. To assess behavior of the general French population, these questions were also included in a survey conducted by Ipsos Observer (Paris, France), an opinion poll marketing company (Certified ISO 9001: 2000 Bureau Véritas Certification), following the quota method (*16*). Quotas were calculated on the basis of sex, age (>15 years), occupation, and location, according to the French National Institute of Statistics and Economic Studies. Interviews were conducted on January 7, 2008. The sample size included 517 persons.

Each questionnaire contained data about age, sex, and socioprofessional characteristics and "commune" (smallest French administrative unit) of living. Nine factors were taken into account for analysis: the 6 above questions, occupation (previous occupation for retired persons), living in rural setting or not, and living in a *département* (second largest administrative unit in France after province) located in an area where persons were at risk for AE (DAR). A *département* where a cluster of cases occurred, identified with SaTScan software (Kulldorff, Boston, MA, USA, and Information Management Services, Inc., Rockville, Md, USA) (*17*), was considered a DAR. SaTScan moved an elliptic window of increasing diameters over the studied region (maximum size allowed for the smallest diameter was 120 km, whereas the largest diameter was not limited) and compared the observed AE case numbers in the window with the expected number under the null hypothesis, that is, the random location of cases among all *départements.* Statistical significance was obtained through Monte Carlo testing (results of the likelihood function were compared with 999 random replications of the dataset generated under the null hypothesis, following Kulldorff's approach) (*18*,*19*).

Data were recorded anonymously by using ACCESS 2000 (Microsoft Corp., Bellevue, WA, USA). Data obtained from the Ipsos Observer survey were weighted to reduce the bias due to the quota sampling. Incidence rates were referring to the mean between 1990 and 1999 national census. For univariate analyses, the Wilcoxon test and Fisher exact test were used, respectively, for quantitative and qualitative variable analysis.

Classification and regression tree (CART) multivariate analysis was used to identify behavioral groups related to AE risk. CART is a nonparametric and nonlinear regressive

approach developed in the 1980s by L. Breiman (*20*,*21*). CART classified persons according to the outcome binary variable—AE patient/non–AE patient. Among all covariates, CART analyzed each possible threshold to split the sample in 2 opposite homogeneous groups. This process was recursively repeated until an optimal criterion was reached.

When only the main covariates were kept, the process enabled a tree to be built in which the terminal classes were groups with common behavior. Because behavior covariates have been known to share collinearity (*22*), CART led to the building of behavioral groups that avoid this bias. To quantify the relationship between the behavioral classes and the disease, we estimated odd ratios (ORs) using a logistic regression. Statistical tests and CART analysis were performed by using R2.13.0 (R Foundation for Statistical Computing; http://cran.r-project.org/).

Results

Département of residence could be accurately determined for 399 of the 407 patients identified by the FrancEchino Network. SaTScan identified 5 significant high risk clusters (p<0.001for each cluster), including 22 *départements*. These 5 clusters have been gathered into 2 separate at-risk areas, an area located in eastern France and the other in the Massif Central, where 84% and 10% of the total of the French AE patients, respectively, are found (Figure 1). Taken altogether, they included 8,900,000 inhabitants, representing 15% of the French metropolitan mean population for the period. In these areas where persons were at risk, the risk of contracting AE was particularly high compared to the risk in the rest of metropolitan France (relative risk 78.63, 95% CI 52.84–117.02).

Because many patients who received a diagnosis of AE during the 1980s and the 1990s were lost to follow-up or had died, only part of the cohort could be interviewed. We obtained 180 questionnaires that could be analyzed, which showed 111 confirmed, 61 probable, and 8 possible cases. Interviewed patients were not significantly different (p>0.289) in terms of diagnosis status, age, sex, occupation, region of living, and type of *commune* from the whole set of patients, except that they were more likely to have been diagnosed after 1994 (p = 0.025).

Of these patients, 164 (91%) were still living in a DAR (Figure 1), 163 (91%) were living in the same *département* as they did 15 years before (144 [80%] in the same village) and 121 (67%) spent their entire life in the same *département*. Seven patients (4%) used to live in a DAR but had left it at diagnosis time for a median time of 11 years (95% interquantile central interval 0.6–26.8). All 7 used to come back into DARs every year to spend holidays. Altogether, the 171 patients who had lived in DARs stayed a median time of 56 years (95% interquantile central interval 12.7–81.5) in DARs before the AE diagnosis was made.

Of the 9 remaining patients who never lived in a DAR, 3 were living <50 km from the border of a DAR, 4 often traveled to DARs for summer holidays, and only 2 had little or no contact with DARs. The first person, who lived in Normandy (northwestern France), spent only 3 months in a DAR for military duty and had not been back for 34 years, and the second person, who lived in northern France, occasionally traveled to DARs, but only for skiing during winter vacations.

Once data were weighted, the IPSOS Observer–interviewed group (controls) accounted for 566 persons. It differed from the whole patient group by mean age (OR 44.9, 95% CI 43.36–46.35 vs. 58.09, 95% CI 56.78–59.40, p<0.001) and geographic location (only 16% living in a DAR vs. 94%, OR 77.92, 95% CI 48.32–131.04, p<0.001) but not for sex ratio (0.92 vs. 1.07; p = 0.27). Univariate analysis showed a significant difference (p<0.001) between interviewed patients and controls for all of the following factors: agricultural occupation (35% vs. 5%; OR 9.58, 95% CI 5.82–16.06), living in DAR (91% vs. 16%; OR 52.37, 95% CI 29.60–98.59) and in rural communes (communes with continuous dwellings with <2,000 inhabitants, 60%

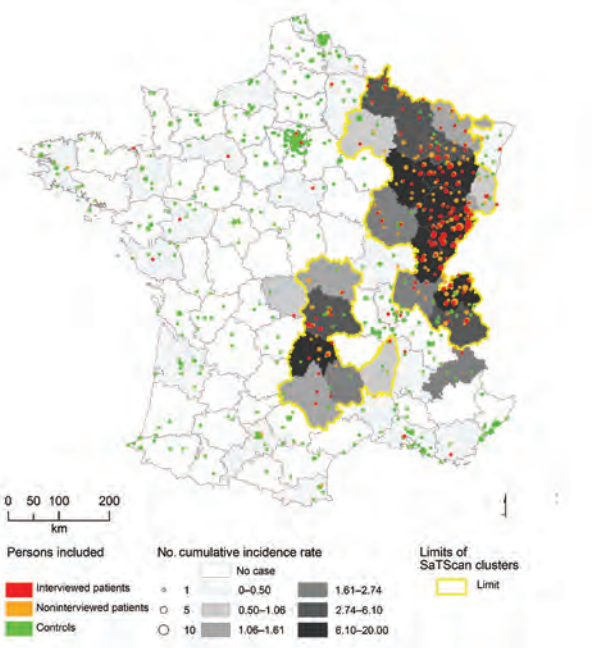

Figure 1. Location of patients, controls, and areas in France where persons are at risk for alveolar echinococcosis. The main area for human risk is located in eastern France and includes the *départements* (second largest administrative areas in France) where persons are at risk for alveolar echinococcosis of clusters 1, 2, and 4 as defined by SatScan analysis (Kulldorff, Boston, MA, USA, and Information Management Services, Inc., Rockville, MD, USA). Clusters 3 and 5 are located in the mountains of Massif Central and constitute the second area where persons are at risk.

vs. 25%; OR 4.51, 95% CI 3.13–6.54), having a kitchen garden (92% vs. 63%; OR 6.98, 95% CI 3.91–13.39), eating raw salads (77% vs. 55%; OR 2.71,. 95% CI 1.83–4.08), having handled a fox at least once (29% vs. 13%; OR 2.69, 95% CI 1.76–4.09), or having a dog (81% vs. 62%; OR 2.59, 1.70–4.03). By contrast, the proportion of hunters did not differ significantly between persons with AE and controls (17% vs. 14%; OR 1.21, 95% CI 0.74–1.95, p = 0.4) and those in the AE group were equally likely to eat raw wild berries as controls (91% vs. 91%; OR 1.04, 95% CI 0.57–2.04, p = 1). The subgroup analysis that compared patients to controls inside DARs showed similar results (Table 1).

Multivariate analysis with CART (Figure 2) led to a definition of 5 classes of persons with a significant level of risk. The class with the lower risk corresponded to all persons living outside the 22 DARs previously defined, whatever their habits, occupation, and site where they used to live (rural or urban). Compared with our reference class (persons living in nonrural communes inside DARs and having no kitchen garden), they exhibited low risk (OR 0.097, 95% CI 0.039–0.250, p<0.001, Table 2).

Among persons who lived in a DAR, having a kitchen garden was associated with an increasing risk for persons who lived in nonrural communes (OR 4.30, 95% CI 1.82–10.91, p = 0.004). However, the risk was higher for persons living in rural communes (OR 6.98, 95% CI 2.88–18.25, p<0.001), and among them, even higher for those with agricultural occupations (OR 66.67, 95% CI 6.21–464.51, p = 0.002).

Discussion and Conclusions

Although it has long been known that most of eastern France and, at a lesser degree, the Massif Central are major foci of AE in Europe (*7*), few data have been available concerning the specific risk factors for this disease in the French population. Our study confirmed that almost all patients with AE live in rural areas in eastern and central France. In addition, a heterogeneous geographic distribution of AE has been reported in numerous foci, such as in Hokkaido, Japan, where humans cases spread from the various areas where infected foxes were introduced in the 1920s (*23*); in the People's Republic of China, where the disease is endemic to only the western and northern provinces and the autonomous regions (*24*); and in other European countries such as Poland, Germany, Austria, and Switzerland (*7*).

In our study, mapping the locations of almost all patients in France who received a diagnosis of AE over 26 years and detecting clusters of patients allowed us to accurately identify the current areas in France where human AE is endemic. Less than one tenth (17/180) of the patients who were interviewed did not live in the same *département* at diagnosis and 15 years before, and only 5% (9/180) had never lived in the DARs defined by our spatial analysis. Taking into account the rarity of the disease in France (15 new patients/year until 2007) (*12*,*13*), this means that <1 patient per year received a diagnosis of AE among 48 million French citizens residing outside DARs during the study period (mean French metropolitan population was 57,500,000 for the study period, with only 8.9 million living in DARs). This low incidence of AE in most regions of France may have been exaggerated by misdiagnosis in areas where the disease is poorly known by physicians. However, the chronicity and the severity of the disease associated with the performances of diagnostic imaging and serologic testing, which greatly improved during the last decades, make a persistent misdiagnosis of AE lesions unlikely (*25*).

Because regulations in France require that albendazole be dispensed only in public hospital pharmacies, we can be reasonably confident that our detection of cases, using a multidisciplinary approach, was complete. Therefore, the clustering of almost all French cases in a few specific locations demonstrates the importance of the place where persons live and had lived in the risk assessment of AE and the precautions that have to be taken when generalizing incidence numbers to the total population. Human data

Table 1. Univariate analysis of studied behavior and area of living and risk for alveolar echinococcosis, France, 1982–2007*

	Lived in DAR				Lived outside DARs			
Variable	No. (%) patients	No. (%) controls	OR (95% CI)	p value	No. (%) patients	No. (%) controls	OR (95% CI)	p value
Total no.	164	92			16	474		
Had agricultural occupation	**62 (38)**	**7 (8)**	**7.33 (3.13–20.00)**	**<0.001**	1 (6)	23 (5)	1.31 (0.03–9.25)	0.558
Had kitchen garden	**152 (93)**	**64 (70)**	**5.50 (2.52–12.66)**	**<0.001**	**14 (88)**	**292 (62)**	**4.35 (0.98–39.90)**	**0.037**
Lived in rural/urban commune	**105 (64)**	**27 (29)**	**4.26 (2.39–7.75)**	**<0.001**	3 (19)	114 (24)	0.73 (0.13–2.72)	0.772
Had dog	**133 (81)**	**56 (61)**	**2.75 (1.49–5.09)**	**<0.001**	13 (81)	297 (63)	2.58 (0.70–14.30)	0.187
Handled fox	**50 (30)**	**12 (13)**	**2.91 (1.42–6.41)**	**0.002**	3 (19)	64 (14)	1.48 (0.26–5.58)	0.469
Ate raw wild salads	128 (78)	61 (66)	1.80 (0.98–3.31)	0.054	10 (63)	249 (53)	1.50 (0.49–5.12)	0.459
Went hunting	28 (17)	15 (16)	1.06 (0.51–2.27)	1	2 (13)	65 (14)	0.90 (0.10–4.06)	1
Ate raw wild berries	149 (91)	84 (91)	0.95 (0.33–2.50)	1	15 (94)	430 (91)	1.53 (0.23–66.05)	1

*DAR, *département* (second largest administrative area in France) located in at-risk areas; OR, crude odds ratio; commune, smallest administrative unit in France. **Boldface** indicates statistical significance.

reflect the parasitic transmission a few years before diagnosis; they are not sufficient to determine the current situation regarding the risk of transmission and must be combined with frequently updated animal data.

In contrast with the highly clustered location of human patients, the main hosts involved in the life cycle of AE (foxes, dogs, voles) are found almost everywhere in France (*26*,*27*). Until the end of the 20th century, infected foxes had never been observed—but also not systematically searched for—outside the areas to which AE is endemic in humans, that is, eastern France from the northeastern border to the southern Alps with a limited focus in Massif Central (the Cantal area) (*26*). Our findings fit well with such a distribution of the infected foxes, although they show a substantial extension of the Massif Central focus toward the southeast and northeast. However, a recent screening campaign of the fox population showed the existence of infected foxes in northwestern France, including in Paris and its suburbs, and reaching the English Channel coast (Normandy region) (*28*). In our series, 2 patients from northern and northwestern France were found to be infected although they had almost never traveled to DARs. Even though AE prevalence in foxes is usually lower outside DARs than in them (leading to a lower risk for humans), these 2 patients could be an early indication of a broader extension of AE in the French population.

Notably, AE lesions develop slowly in humans, leading to a long period of latency between the initial infection and the diagnosis. Because of this latency period, AE in humans would increase only 10–15 years after *E. multilocularis* infection incidence increased in foxes. Such a phenomenon has already been observed in Switzerland (*10*), and recently Takumi et al. hypothesized that this could happen in the Netherlands before 2020 (*29*). Moreover, foxes are now often living in urban areas (*30*–*32*) and have a high incidence rate of *E. multilocularis* infection in some cities located in AE-endemic areas (*30*), so urban gardeners could have a higher risk in the future. Prevention campaigns must target this group in addition to rural inhabitants in regions where AE is endemic in humans and foxes.

Our findings identified many factors associated with patients' way of life such as having an agricultural occupation or having a kitchen garden, 2 factors that increase contact with soil possibly contaminated by *E. multilocularis* eggs. Having a dog and/or handling foxes, 2 risk factors associated with the spread of *E. multilocularis* eggs by their main definitive hosts, were also significant variables. In Germany, a case-control study also found that farmers were at highest risk (*22*), whereas gardeners were found to have additional risk only if they grew leafy or root vegetables.

The most commonly alleged source of infection emphasized in public media, that is, "eating wild berries,"

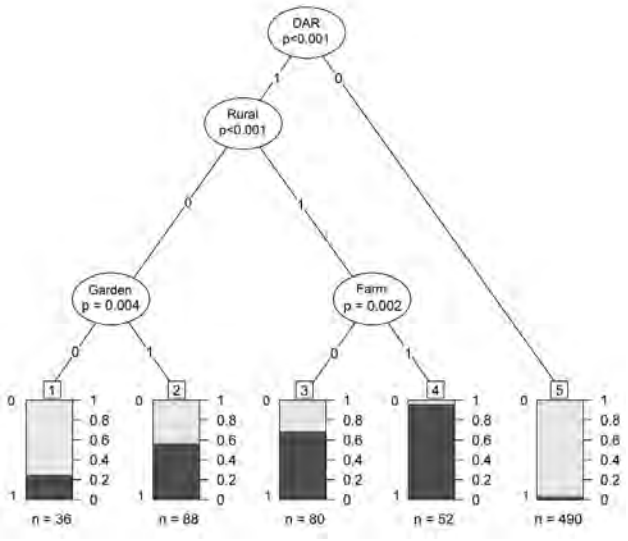

Figure 2. Significant results by multivariate analysis using classification and regression tree analysis to determine risk for alveolar echinoccosis in France, 1982–2007. Black indicates patients; gray indicates controls; class number is enclosed in a square. When the last step of analysis was not significant, terminal classes were aggregated at the upper level. Patients appeared predominant in 4 terminal classes: class 1 represented persons who live in an urban (or semiurban) environment in a *département* (second largest administrative area in France) where persons are at risk for alveolar echinococcosis (DAR) and do not have a kitchen garden, class 2, persons who live in similar areas but have a kitchen garden; class 3, nonfarmers who live in rural areas in a DAR; and class 4, farmers who live in the same environment; class 5, mostly persons who live in *départements* (second largest administrative areas in France) where humans are not at risk.

was not found to be an additional risk in our study. Conversely, we found that almost everyone did pick berries (91% in both the control and AE groups). If such behavior would have been a noticeable risk factor, some cases should have been diagnosed every year among the millions of the French city dwellers who go to DARs for hiking and tourism. In Germany, Kern et al. (*22*) could not rule out the category "eating unwashed strawberries" when building an individual risk score for AE. However, this factor was the least significant included in the score they developed. Similarly, our findings, as well as those of Kern et al., did not link AE with hunting. In contrast, a previous study in Austria (*33*) identified hunting as the most notable observed risk factor (OR 7.83, 95% CI 1.16–52.77). This Austrian study included only 21 cases, preventing he authors from analyzing confounding factors such as living in a rural area. This study did not find that owning a dog led to a statistical risk.

Dog ownership has been noted in many countries (Saint-Laurent Island, Alaska, USA [*34*], China [*35*–*37*],

Table 2. Analysis of behavioral classes and risk for alveolar echinococcosis, France, 1982–2007*

Class no.†	OR (95% CI)
1	1
2	4.30 (1.82–10.91)
3	6.98 (2.88–18.25)
4	66.67 (6.21–464.51)
5	0.097 (0.039–0.250)

*OR, odds ratio; DAR, *département* (second largest administrative area in France) where persons are at risk for alveolar echinococcosis.
†When the last step was not significant, classes were aggregated at the upper level. Class 1 is the reference class: persons living in an urban (or semiurban) environment, in a DAR and having no kitchen garden; class 2, persons living the same environment in DAR but having a kitchen garden; class 3, nonfarmers living in rural settings, in DAR; class 4, farmers living in the same environment; class 5, persons living in *départements* where persons were not at risk.

and Turkey [*38*]). In Germany (*22*), this was a major risk only when persons owned dogs that "roamed outside," "killed game," or "were irregularly dewormed." Overall, CART analysis was of particular interest in our process to determine AE risk factors. It is obvious that a person living in a rural area in France is more likely to own a dog, have a kitchen garden, and work in agriculture than a person living in a city. Although the method only indicates broad trends, CART could bypass the problem of collinearity between variables and allow the main profiles of persons to be defined on the basis of specific level of risk of infection.

In conclusion, determining who is at risk of acquiring AE will enable prevention campaigns to be focused on specific population groups. Tools and recommendations are already available to limit the risk of infection in humans (*23*). These tools include frequent hand washing, proper food handling, pet deworming, discontinuing vegetable gardens, and avoiding contact with foxes. Using bait to deworm foxes has also been proposed (*30*). Because AE remains mostly clustered in geographically distinct areas (and persons who spend only vacation time in these AE-endemic areas do not appear at high risk), sensitization campaigns should be aimed at persons who live in AE-endemic areas, especially those who have an agricultural occupation, have a garden, or live in rural settings. Local outlets, such as rural medical offices, pharmacies, and communal administrative information bulletins as well as local newspapers and radio and TV channels could convey awareness messages. Placing general advertisements in national media might be counterproductive by alarming persons not at risk for the disease and by focusing on the most sensational or supersensitive information (such as the potential danger of wild berries) (*39*,*40*), while overlooking more obvious measures to avoid contamination, such as pet deworming and hand washing after gardening or playing with pets. Nevertheless, the situation might change, and screening for human AE cases and continuous monitoring of fox infections could enable this strategy to be adapted to new foci as necessary.

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World Health Organization International Standard to Harmonize Assays for Detection of Hepatitis E Virus RNA

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Nucleic acid amplification technique–based assays are a primary method for the detection of acute hepatitis E virus (HEV) infection, but assay sensitivity can vary widely. To improve interlaboratory results for the detection and quantification of HEV RNA, a candidate World Health Organization (WHO) International Standard (IS) strain was evaluated in a collaborative study involving 23 laboratories from 10 countries. The IS, code number 6329/10, was formulated by using a genotype 3a HEV strain from a blood donation, diluted in pooled human plasma and lyophilized. A Japanese national standard, representing a genotype 3b HEV strain, was prepared and evaluated in parallel. The potencies of the standards were determined by qualitative and quantitative assays. Assay variability was substantially reduced when HEV RNA concentrations were expressed relative to the IS. Thus, WHO has established 6329/10 as the IS for HEV RNA, with a unitage of 250,000 International Units per milliliter.

Hepatitis E virus (HEV) is a nonenveloped, single-stranded RNA virus belonging to the family *Hepeviridae* (*1*,*2*). In developing countries, HEV is a major cause of acute hepatitis, transmitted by the fecal–oral route and associated with contamination of drinking water. In industrialized countries, reports of HEV infection have been uncommon but are being reported more frequently; some cases are imported after travel to HEV-endemic areas, but reports of autochthonous cases are also increasing, and infection with HEV appears to be more prevalent than originally believed (*3*). Prospects for control of HEV infection are encouraged by recent efforts in vaccine development (*4*,*5*).

Four main genotypes of HEV, representing a single serotype, infect humans. Genotype 1 viruses are found mainly in Africa and Asia and genotype 2 in Africa and Central America; it is in these areas that prevention of HEV infection by vaccination would be most beneficial. Genotypes 3 and 4 viruses are generally less pathogenic, although some exceptions have been reported, particularly for genotype 4; these genotypes infect not only humans but also animals such as swine, wild boar, and deer. Although genotype 4 strains have mainly been restricted to parts of Asia, genotype 3 viruses are found widely throughout the world. Zoonotic transmission of HEV genotypes 3 and 4 to humans can occur by consumption of contaminated meat or meat products or by contact with infected animals (*6*,*7*). Shellfish, such as bivalve mollusks, have also been shown to act as reservoirs for HEV (*8*).

An alternate route of transmission of HEV by transfusion of blood components has been reported in Japan (9,10), the United Kingdom (11), and France (*12*,*13*). Studies in Japan (*14*) and the People's Republic of China (*15*) have identified acute HEV infections in blood donors, confirmed by the detection of HEV RNA. Analysis of blood and plasma donors in Europe has identified HEV-infected donors in Germany (*16*–*20*), Sweden (*18*), and England (*21*). Transmission of HEV by solid organ transplantation has also been reported (*22*). Rates of HEV infection may be underreported in some countries, and misdiagnosis of HEV infection also occurs. For example, in some cases of suspected drug-induced liver injury, HEV has been determined

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[1]Members of the HEV Collaborative Study Group are listed at the end of this article.

[2]In memory of Thomas Laue.

as the cause (*23*). In one such recent case, HEV was shown to have been transmitted by blood transfusion (*13*).

Infection with HEV may cause particularly severe illness in pregnant women and in persons who have preexisting liver disease. Chronic infection with HEV genotype 3 is an emerging problem among solid organ transplant recipients and may also occur in persons with HIV and certain hematologic disorders (*24*). In patients with chronic infection, viral loads are monitored to investigate the efficacy of antiviral treatment (*25*,*26*) and effects of reduction of immunosuppressive therapy (*27*).

HEV infection is diagnosed on the basis of detection of specific antibodies (IgM and IgG), but the sensitivity and specificity of these assays is not optimal (*28*–*30*). Analysis of HEV RNA by using nucleic acid amplification techniques (NATs) is also used for diagnosis; this method can identify active infection and help confirm serologic results (*31*). Several NAT assays have been reported for the detection of HEV RNA in serum and plasma or fecal samples: conventional reverse transcription PCR (RT-PCR) and nested protocols (*32*), real-time RT-PCR, and reverse transcription loop-mediated isothermal amplification (*33*). The NATs include generic assays designed for the detection of HEV genotypes 1–4 (*34*,*35*).

In 2009, the World Health Organization (WHO) Expert Committee on Biological Standardization endorsed a proposal by the Paul-Ehrlich-Institut (PEI) to prepare an International Standard (IS) for HEV RNA for use in NAT-based assays. PEI recently completed an initial study that investigated the performance of HEV NAT assays in detection of HEV infection (*36*). In that study, dilution panels of HEV genotype 3 and 4 strains underwent blinded testing in laboratories that had experience in detection of HEV RNA. Results demonstrated wide variations in assay sensitivity (in the order of 100- to 1,000-fold for most assays).

After the initial study, 2 virus strains included in the panel (*36*) were selected for further development of a candidate IS for the WHO, and a candidate Japanese national standard (done in collaboration with the National Institute of Infectious Diseases in Tokyo). These viruses belong to genotype 3, which is widely distributed, and were genotype 3a and 3b strains, which were equally well detected in the initial study. The strains were derived from plasma samples that had sufficient titers of HEV RNA to prepare standards of good potency. An international collaborative study was conducted to establish the respective standards, demonstrate suitability for use, evaluate potency, and assign an internationally agreed-upon unitage.

Methods

Preparation of Materials

The 2 HEV strains selected for the preparation of the candidate WHO IS and candidate Japanese national standard were genotype 3a strain HRC-HE104 and genotype 3b strain JRC-HE3, respectively. The HEV-positive plasma donations were kindly provided by the Japanese Red Cross Society Blood Service Headquarters (Tokyo, Japan). Characterization of the stock virus strains is shown in Table 1.

The samples were tested for IgG/IgM against HEV by using an HEV enzyme immunoassay (Institute of Immunology Co., Ltd., Tokyo, Japan). Full-length sequences of the HEV strains were determined as described (*37*). Phylogenetic analyses were conducted by using MEGA version 5.05 (*38*), and HEV genotype and subgenotype were determined as described (*39*). The nucleotide sequences of HRC-HE104 and JRC-HE3 were deposited into GenBank under accession nos. AB630970 and AB630971, respectively.

The target HEV RNA concentration for the 2 bulk standard preparations was ≈5.5 $\log_{10}$ HEV RNA copies/mL, on the basis of the concentrations determined in the initial study (*36*). The 2 virus strains were negative when tested for hepatitis B virus, hepatitis C virus, and HIV-1/2 by using the Cobas TaqScreen MPX test (Roche Molecular Systems Inc., Branchburg, NJ, USA). The samples were diluted by using pooled citrated plasma (*36*) that had tested negative by NAT for hepatitis B virus, hepatitis C virus, and HIV-1/2, and HEV and was also negative for antibodies against HEV by using the recomWell IgG and IgM enzyme immunoassays (Mikrogen GmbH, Neuried, Germany). The diluted plasma was placed into 4-mL screw-cap glass vials, freeze dried, filled with nitrogen, sealed with rubber stoppers, and stored at –20°C. Stability studies demonstrated no substantial change in HEV RNA concentration after freeze drying or after 10 months of storage at –20°C (the usual temperature), +4°C, and +20 to +26°C, compared with samples stored at ≤–80°C.

Table 1. HEV strains diluted and lyophilized as candidate standards in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Virus strain	HEV RNA, copies/mL	Genotype	GenBank accession no.	IgM/IgG against HEV	Alanine aminotransferase, IU/L
HRC-HE104	1.6×10^7	3a	AB630970	–/–	36
JRC-HE3	2.5×10^7	3b	AB630971	+/–	398

*Strains were provided by the Japanese Red Cross Society Blood Service Headquarters, Tokyo, Japan. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique.

Study Design

The collaborative study was conducted by 24 laboratories from 10 countries; each laboratory was randomly assigned a code number. The samples analyzed in the study were coded sample 1 and sample 2 (replicates of the candidate WHO IS) and sample 3 and sample 4 (replicates of the candidate Japanese national standard). Samples were shipped to participants at ambient temperature. Participants tested the samples by using the laboratory's routine assays for HEV RNA, in 4 separate assay runs, using fresh vials of each sample for each run. Quantitative assay results falling within the linear range of the assays were reported in copies/mL. For qualitative assays, participants assayed each sample by a series of 1.0-$\log_{10}$ dilution steps to obtain an initial estimate of an endpoint and then, in 3 subsequent runs, assayed 0.5-$\log_{10}$ dilutions around the endpoint determined in the first run.

Statistical Methods

Quantitative Assays

Evaluation of quantitative assays was restricted to dilutions of 0.0 $\log_{10}$ to -2.5 $\log_{10}$, a range over which the assays of most participants produced comparable data. For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as the arithmetic mean of $\log_{10}$ copies/mL. Furthermore, these estimates were combined to obtain an overall estimation for each sample by means of a mixed linear model, using laboratory and $\log_{10}$ dilution as random factors.

Qualitative Assays

The data from all assays were pooled to give a series of values for number positive/number tested at each dilution. For each participant, these pooled results were evaluated by means of probit analysis to estimate the concentration at which 50% of the samples tested were positive; for assays in which the change from complete negative to complete positive results occurred in ≤2 dilution steps, the Spearman-Kaerber method was applied for estimation. The calculated endpoint was used to give estimates expressed in $\log_{10}$ NAT-detectable units/mL, after correcting for the equivalent volume of the test sample.

Relative Potencies

For quantitative assays, potencies of samples 2, 3, and 4 were estimated relative to sample 1 by using parallel-line analysis of log-transformed data. For qualitative assays, relative potencies were determined by using parallel-line analysis of probit-transformed data. Statistical analyses were performed by using SAS/STAT version 9.3 (SAS Institute, Cary, NC, USA). Estimation of endpoint dilution and relative potencies was performed by using CombiStats version 4.0 (European Directorate for the Quality of Medicines and HealthCare/Council of Europe, Strasbourg, France).

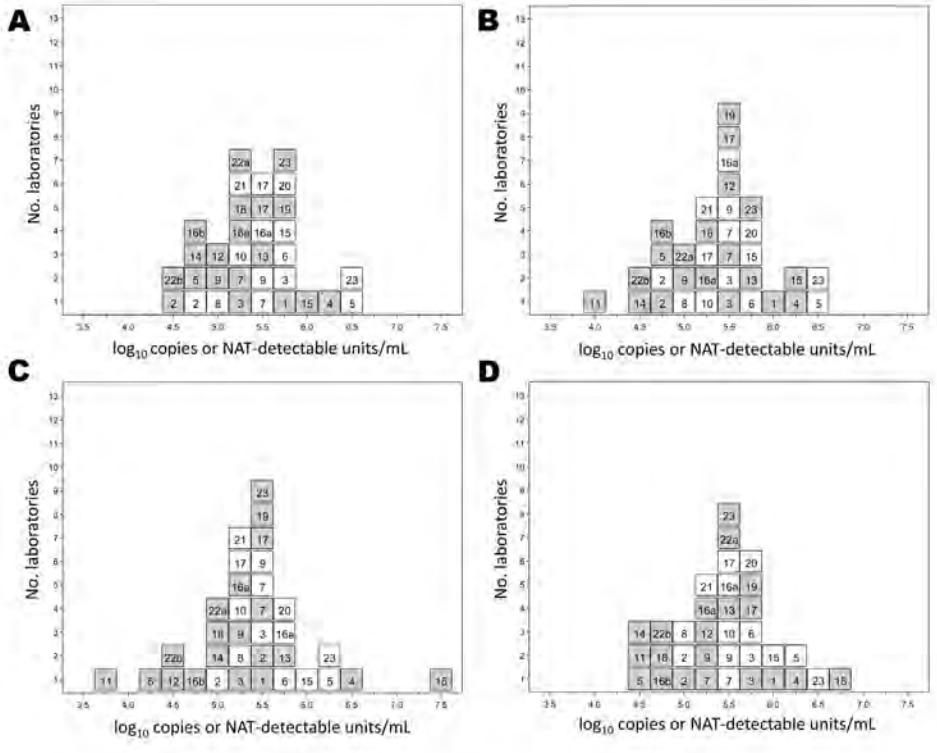

Figure 1. Histograms showing results for quantitative and qualitative assays conducted by 23 laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). White indicates quantitative assays ($\log_{10}$ copies/mL); gray indicates qualitative assays ($\log_{10}$ nucleic acid amplification technique (NAT)–detectable units/mL). Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes.

Results

Data were returned by 23 of the 24 participating laboratories; 20 sets of qualitative data and 14 sets of quantitative data were evaluated. The assays used by the participants are shown in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/5/12-1845-Techapp1.pdf). All assays were developed in-house and were either conventional or nested RT-PCRs or based on real-time RT-PCR.

Quantitative and Qualitative Assay Results

Laboratory mean estimates for quantitative assays (in $\log_{10}$ copies/mL) and qualitative assays (in NAT-detectable $\log_{10}$ units/mL) for the HEV preparations are shown in histogram form in Figure 1, which shows that laboratory means are more variable for the qualitative assays than the quantitative assays, reflecting different assay sensitivities and lack of standardization. The individual laboratory means are given in online Technical Appendix Tables 2 and 3; relative variation of the individual laboratory estimates for the quantitative assays is illustrated by the box-and-whisker plots in Figure 2. Intralaboratory variation was lower than the interlaboratory variation for both types of assays (data not shown).

Determination of Overall Laboratory Means

The means for all the laboratories performing quantitative assays are shown in Table 2. The means for sample 1 and sample 2, replicates for the candidate WHO IS, were 5.58 $\log_{10}$ and 5.60 $\log_{10}$ copies/mL HEV RNA, respectively, with good agreement between the replicate samples. The candidate Japanese national standard showed identical mean results of 5.66 $\log_{10}$ copies/mL HEV RNA for replicate samples 3 and 4.

The means for all the laboratories performing qualitative assays are also shown in Table 2; again, there was good agreement between the duplicate samples. Results for the qualitative assays showed 0.3-$\log_{10}$ lower mean estimates and a higher SD than those for the quantitative assays. The combined mean values for the replicate samples for both types of assays are shown in Table 2.

Relative Potencies

On the basis of the combined data from both qualitative and quantitative assays, the candidate WHO standard was determined to have a potency of 5.39 $\log_{10}$ units/mL (95% CI 5.15–5.63). This value was calculated with a combined endpoint evaluation of qualitative and quantitative data (restricted to dilutions in the range of 0.0 $\log_{10}$ to -2.5 $\log_{10}$) by means of a mixed linear model.

The potencies of samples 2, 3, and 4 were calculated relative to sample 1, taking the value of sample 1 as 5.39 $\log_{10}$ units/mL. The relative potencies for the quantitative and qualitative assays are shown in online Technical Appendix Tables 4 and 5, respectively. Table 3 summarizes the overall mean potencies relative to sample 1, with the 95% CIs, SDs, and geometric coefficients of variation. For the quantitative data from laboratory 9, no potency could be estimated by endpoint evaluation because only 1

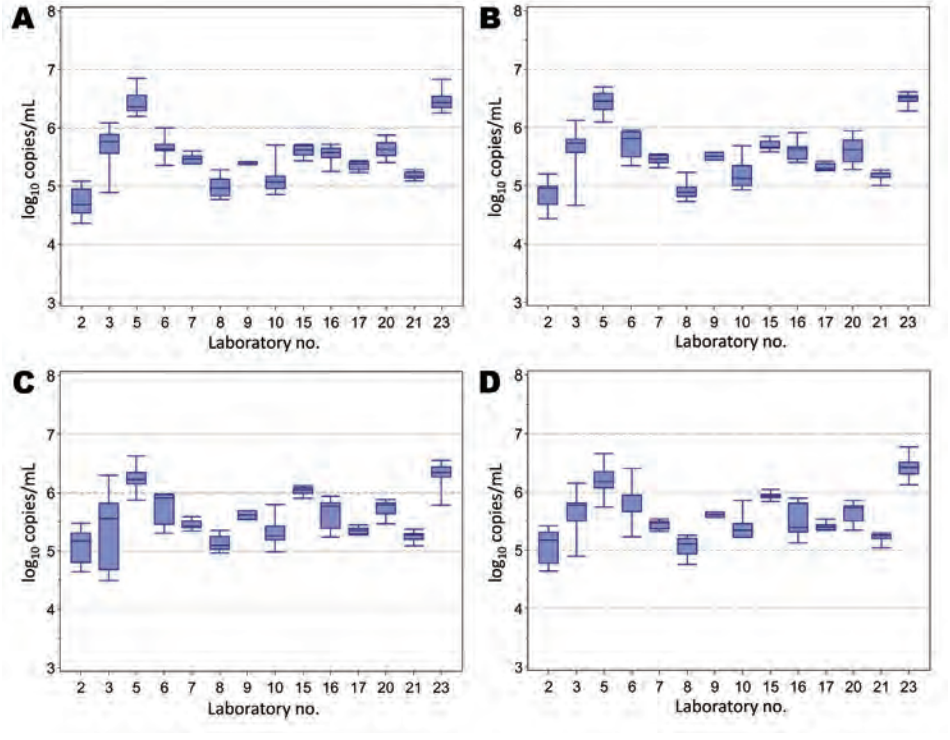

Figure 2. Box and whisker plots of the results for quantitative assays ($\log_{10}$ copies/mL) conducted by laboratories for the determination of the hepatitis E virus (HEV) RNA content of sample 1 (A), sample 2 (B), sample 3 (C), and sample 4 (D). Box indicates interquartile range; line within box indicates median; whiskers indicate minimum and maximum values observed. Laboratory code numbers are given on the horizontal axis.

Table 2. Overall mean estimates from quantitative and qualitative assays of HEV samples in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Assay type and sample	No.	Mean (95% CI)†	SD	% CV
Quantitative				
1	123	5.58 (5.32–5.85)	0.54	98
2	125	5.60 (5.33–5.87)	0.53	94
1 + 2	248	5.59 (5.33–5.86)	0.55	99
3	124	5.66 (5.40–5.93)	0.45	77
4	125	5.66 (5.40–5.93)	0.44	76
3 + 4	249	5.66 (5.40–5.93)	0.44	76
Qualitative				
1	19	5.25 (5.01–5.50)	0.51	150
2	20	5.26 (4.97–5.56)	0.62	179
1 + 2	39	5.26 (5.08–5.44)	0.56	163
3	20	5.27 (4.90–5.64)	0.79	226
4	20	5.31 (5.02–5.61)	0.64	183
3 + 4	40	5.29 (5.07–5.52)	0.71	202

*Samples 1 and 2, replicate samples of the candidate WHO International Standard; samples 3 and 4, replicate samples of the candidate Japanese national standard. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique; no., no. dilutions analyzed (in linear range for quantitative assays); % CV, geometric coefficient of variation.
†Values are $\log_{10}$ copies/mL for quantitative and $\log_{10}$ NAT–detectable units/mL for qualitative assays.

dilution was tested for each sample. The data are plotted in histogram form in Figure 3.

The data demonstrate that expressing the results as potencies relative to sample 1 (set as a standard with an assumed unitage of 5.39 $\log_{10}$ units/mL) results in a marked improvement in the agreement between the majority of methods and laboratories, as evidenced by the reduction in SDs. Furthermore, these data provide some evidence for commutability of the candidate standard for evaluation of HEV from infected persons, because samples 1 and 2 represent a different strain of HEV compared with samples 3 and 4.

Discussion

In this study, a wide range of quantitative and qualitative assays were used to determine the suitability and evaluate the HEV RNA content of the candidate standards. Although the methods used by the study participants were all developed in-house, most assays consistently detected the 2 HEV strains. On the basis of data from the qualitative and quantitative assays, the candidate WHO IS was estimated to have a potency of 5.39 $\log_{10}$ units/mL. For practical purposes, the candidate IS was assigned a unitage of 250,000 International Units (IU)/mL; because the difference in the overall mean for the candidate Japanese national standard was negligible compared with the WHO preparation, the 2 materials were assigned the same value. In the case of the quantitative assays, laboratories reported values in HEV RNA copies/mL. The participating laboratories used plasmid DNA containing HEV sequences, synthetic oligonucleotides, and in vitro–transcribed HEV RNA to control for copy number. In some cases, laboratories used HEV-containing plasma that had been calibrated against in vitro–transcribed HEV RNA. One laboratory prepared a standard by using stool-derived virus, the titer of which was determined by endpoint dilution and analysis by Poisson distribution. No standard method or common quantitation standard material was used; this fact is reflected in the variation observed for the quantitative results (in the order of 2 $\log_{10}$), which were improved by expressing the results against sample 1 as a common standard. For qualitative assays, the variation in NAT-detectable units was $\geq$3 $\log_{10}$, and as with quantitative assays, expressing potencies relative to sample 1 improved the agreement among the different laboratories and methods.

Many of the laboratories participating in the study used a real time RT-PCR developed in 2006 (*34*) that was designed to detect the 4 main genotypes of HEV. However, a recent study in the United Kingdom found a polymorphism in the probe-binding site in several HEV-infected patients who initially had negative test results using this assay (*40*). A modification of the probe, increasing the melting temperature, restored detection of the polymorphic virus strains. We identified a further polymorphism in an HEV strain (GenBank accession no. JN995566) from a plasma donor (*18*), located in the probe-binding site of the same assay; use of the modified probe improved the amplification curve for this virus strain (S. Baylis and T. Gärtner, unpub. data). Genetic variation and its potential effects on HEV RNA detection highlight the importance of confirmatory tests of different design, rather than reliance on single methods.

The WHO IS will be valuable for development of secondary standards traceable to the IU, which will facilitate comparison of results between laboratories and

Table 3. Overall mean potencies of samples 2, 3, and 4 relative to sample 1 from quantitative and qualitative analysis of HEV samples in study to establish a WHO International Standard for HEV RNA NAT-based assays*

Sample and assay type	No.	Mean (95% CI)†	SD	% CV
Sample 2				
Quantitative	19	5.46 (5.35–5.58)	0.23	3
Qualitative	13	5.42 (5.38–5.46)	0.07	1
Combined	32	5.45 (5.38–5.51)	0.18	2
Sample 3				
Quantitative	20	5.45 (5.27–5.65)	0.43	5
Qualitative	13	5.48 (5.37–5.59)	0.18	2
Combined	33	5.46 (5.35–5.58)	0.35	4
Sample 4				
Quantitative	20	5.51 (5.38–5.64)	0.29	3
Qualitative	13	5.47 (5.36–5.59)	0.19	2
Combined	33	5.49 (5.41–5.58)	0.25	3

*Mean potency values were determined by assigning a value of 5.39 $\log_{10}$ units/mL for sample 1. Samples 1 and 2, replicate samples of the candidate WHO International Standard; samples 3 and 4, replicate samples of the candidate Japanese national standard. HEV, hepatitis E virus; WHO, World Health Organization; NAT, nucleic acid amplification technique; no., no. dilutions analyzed (in linear range for quantitative assays); % CV, geometric coefficient of variation.
†Values are $\log_{10}$ copies/mL for quantitative and $\log_{10}$ NAT technique–detectable units/mL for qualitative assays.

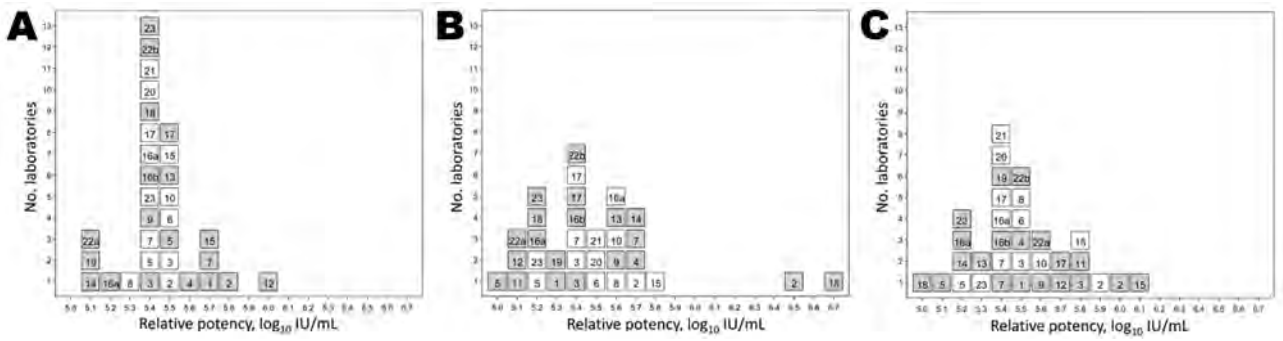

Figure 3. Histograms showing potencies of sample 2 (A), sample 3 (B), and sample 4 (C) compared with sample 1, the candidate World Health Organization International Standard for hepatitis E virus RNA for nucleic acid amplification technique (NAT)–based assays. White indicates quantitative assays (log_{10} copies/mL); gray indicates qualitative assays (log_{10} NAT–detectable units/mL). Number of laboratories is indicated on the vertical axis. Laboratory code numbers are indicated in the respective boxes.

determination of assay sensitivities and be helpful for validation purposes. We anticipate that the IS will find application in clinical laboratories, particularly in hepatitis reference laboratories that perform diagnosis and monitor HEV viral loads in chronically infected patients. The IS will also be helpful for research laboratories and blood and plasma centers that implement HEV NAT screening, regulatory agencies and organizations that are working to develop HEV vaccines, and manufacturers of HEV diagnostic kits.

The established WHO IS has been prepared by using a genotype 3a HEV strain. WHO has further endorsed a proposal by the PEI to prepare a genotype panel for HEV for NAT-based assays to continue standardization efforts for detection of this emerging infection. It is intended that the panel will contain representative strains of the 4 main genotypes of HEV that infect humans and notable subgenotypes. A new collaborative study will evaluate the IS against other genotypes and subgenotypes of HEV and investigate the commutability of the IS for standardization of assays for different genotypes of HEV. Laboratories that are able to provide high-titer HEV samples to aid in development of the proposed panel are requested to contact the authors.

In summary, WHO has established a genotype 3a HEV strain as the IS for HEV RNA (code number 6329/10), with an assigned a unitage of 250,000 IU/mL. The WHO IS for HEV RNA is available from PEI (www.pei.de).

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Dr Baylis is a scientist at the Paul-Ehrlich-Institut. Her work focuses on adventitious viruses in biological medicines, particularly with respect to blood and plasma-derived products.

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Full-Genome Deep Sequencing and Phylogenetic Analysis of Novel Human Betacoronavirus

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A novel betacoronavirus associated with lethal respiratory and renal complications was recently identified in patients from several countries in the Middle East. We report the deep genome sequencing of the virus directly from a patient's sputum sample. Our high-throughput sequencing yielded a substantial depth of genome sequence assembly and showed the minority viral variants in the specimen. Detailed phylogenetic analysis of the virus genome (England/Qatar/2012) revealed its close relationship to European bat coronaviruses circulating among the bat species of the Vespertilionidae family. Molecular clock analysis showed that the 2 human infections of this betacoronavirus in June 2012 (EMC/2012) and September 2012 (England/Qatar/2012) share a common virus ancestor most likely considerably before early 2012, suggesting the human diversity is the result of multiple zoonotic events.

The ability of coronaviruses (CoVs) to infect multiple species and to rapidly change through recombination presents a continuing human health threat. The epidemic of severe acute respiratory syndrome (SARS) during 2003–2004 during which a CoV transmitted from bats to civet cats and then to humans demonstrated this potential (reviewed in [*1*,*2*]). Recently, a novel human betacoronavirus (betaCoV) was found to be associated with at least 13 human infections, 7 of which were fatal (*3*–*7*). One of the viruses, EMC/2012, has been sequenced, and its sequence similarity to several bat CoVs suggested an animal origin, but a definitive bat species of origin has not yet been identified (*3*). Additional genome sequences from this virus are needed to aid diagnostics, monitor population dynamics, identify the animal source, and characterize mechanisms of pathogenesis. The large size (30,000 nt) and high variability of CoV RNA genomes present a challenge for sequencing.

We describe a strategy for rapidly designing the primers necessary for reverse transcription and cDNA amplification of such diverse RNA viruses and report the full-genome determination of the novel CoV directly from patient sputum using next-generation short-read sequencing. Full genomes from 2 epidemiologically unlinked novel CoV infections separated in time by >2 months were analyzed to gain clues to 2 major questions: what are the precursors of the virus, and how long has the virus been circulating in its current form?

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Materials and Methods

Primer Design

Fifteen betaCoV full genomes with the closest homology to EMC/2012 (GenBank accession no. JX869059) were analyzed to identify all possible primer-like sequences (melting temperature 58°C–60°C, guanine plus cytosine content 35%–60%) yielding 357,198 potential primers. The 30-kb JX869059 CoV genome was divided into fifteen 2.5-kb overlapping amplicons, and the 2 highest frequency sequences mapping in the 5′ and 3′ 250 bp of each amplicon were selected. Reverse complements of the 3′ mapping sequences were prepared, resulting in a set of 60 primers for the 15 amplicons (4 primers/amplicon). Three additional primers were added for the extreme termini of the genome. The algorithm also prepared a virtual PCR map of the predicted binding and amplicon size for the primer set on CoV genomes. A map of the primer mapping positions

and the predicted PCR products using EMC/2012 as the target is shown in Figure 1, panel A. The primer sequences and details of their use are listed in the online Technical Appendix Table (wwwnc.cdc.gov/EID/article/19/5/13-0057-Techapp1.pdf).

Sample History

The patient, originally from Qatar, visited Saudi Arabia, where he became unwell with severe respiratory disease and renal failure and was transferred to a hospital in London, UK (*4*). Viral RNA was extracted from a sputum sample collected on September 19, 2012, sixteen days after symptom onset. Real-time PCR was performed by using an assay targeting the *upE* gene (*6*) and confirmed by using primers and probe targeting the RNA-dependent RNA polymerase (*RdRp*) gene. RdRpTaq1: (5′-GAC TAA TCG CCA GTA CCA TCA G-3′), RdRpTaq2: (5′-GAA CTT TGT AGT ACC AAT GAC GCA-3′), RdRpProbe: (5′-FAM-ATG CTT AAG TCC ATG GCT GCA ACT CGT GGA G–BHQ-3′). The assays gave cycle threshold values of 17.83–18.01 for the *upE* and *RdRp* targets. A 100-μL sample of sputum was lysed with the addition of an equal volume of Qiagen Lysis Buffer AL (QIAGEN, Hilden. Germany). RNA was extracted from 200 μL of this material (final elution volume 60 μL) by using the EZ1 Virus Mini Kit v2.0 and EZ1 instrument (QIAGEN).

Reverse Transcription and PCR Amplification

Reverse transcription was performed at 50°C for 60 min by using the amplicon reverse primers. PCRs were then performed with forward and reverse primers for each amplicon (15 reactions) for 35 cycles (98°C, 10 sec; 54.5°C, 30 sec; 72°C, 2.5 min) with a final 10-min 72°C extension. A 3-μL aliquot of each reaction was analyzed by electrophoresis, and each reaction showed the expected 2–2.5-kb products (Figure 1, panel B). A detailed protocol is available on request from the authors.

Sequencing

The PCR products of amplicons were combined into 4 pools of approximately equal molarity and processed into standard Illumina multiplex libraries (Illumina, San Diego, CA, USA) with each pool bearing a unique bar code sequence. Libraries were sequenced by Illumina MiSeq generating 150-bp paired end reads. The resulting Illumina read sets were processed to remove adaptor and primer sequences and quality controlled to ensure a median read Phred quality score of 30 by using QUASR (*8*). The 5′- and 3′-most primers were derived directly from the first 27 nt or last 31 nt of the EMC/2012 genome. These primer sequences were not trimmed from the reads, which might mask sequence difference between EMC/2012 and England/Qatar/2012 at these positions.

Genome Assembly

Reference-based mapping and de novo assembly methods were applied to the data for assembly into viral genomes. Reference-based mapping was performed against the EMC/2012 genome by using the Burrows-Wheeler Aligner software package [*9*]). For de novo assembly, maximum contig lengths were obtained by using subsets of 30,000–60,000 reads. Therefore, random subsets of reads were extracted from the readset and assembled by using Velvet version 1.2.07 (*10*,*11*) and VelvetOptimiser (*12*). The de novo assembled sequences were used to confirm the validity of the reference-based sequence and showed that the de novo assembly and the reference-based mapping produced identical sequences, apart from several small gaps near the termini of the de novo assembled sequence (results not shown). The complete genome sequenced here is named England/Qatar/2012 and is available in GenBank (accession no. KC667074).

Sequence Alignment

The complete England/Qatar/2012 genome was combined with 46 previously published complete genomes of

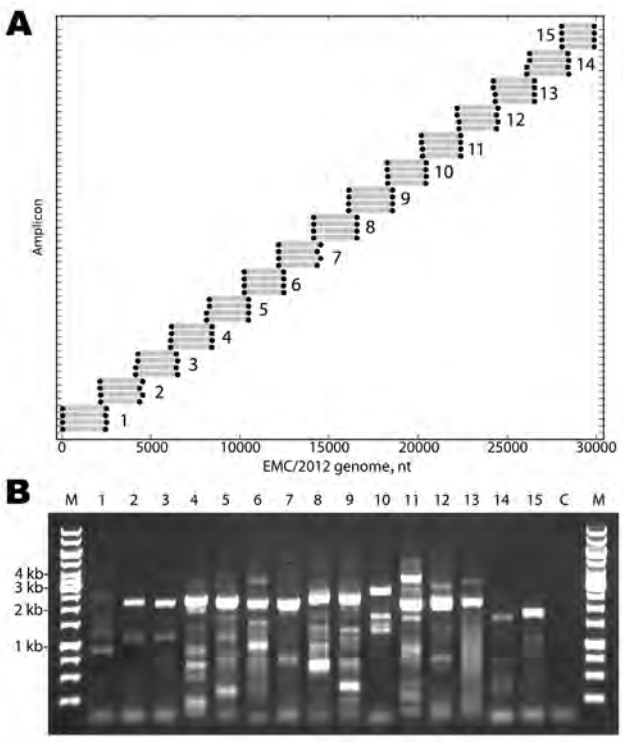

Figure 1. A) Primers designed for reverse transcription and overlapping PCR amplification of the novel coronavirus (CoV). Dots indicate the predicted binding site of each primer along the EMC/2012 genome (x-axis). Gray bars indicate predicted amplicon lengths. Amplicon numbers are indicated beside each set of products. B) PCR products (3 μL of a 25-μL reaction) were resolved by electrophoresis on a 0.6% agarose gel and visualized by ethidium bromide staining. Lane M is the molecular weight marker (sizes indicated at left), Lanes 1–15 show the products of the amplicons depicted in Panel A. Lane C is the reagent PCR control.

the α-(group 1), β-(group 2), γ-(group 3), and δ-(group 4) CoVs. The betaCoV complete genomes encompassed the a, b, c, and d lineages, as well as the 2 genomes of the novel human betaCoV, EMC/2012 (GenBank accession no. JX869059) and England1 (GenBank accession no. KC164505), the latter from the same patient as studied here. The sequences were aligned by using MUSCLE (*13*) within MEGA5 (*14*). Poorly aligned regions and the genes absent in some CoV genotypes were excluded from the alignment, resulting in a virtual concatenation of the open reading frame (ORF) 1ab, S, E, M, and N genes. A second alignment using a 396-bp region of the *RdRp* was generated similarly. This shorter alignment included strains that are genetically close to England/Qatar/2012 but lack complete genome sequences in GenBank.

Phylogenetic Methods

Maximum-likelihood (ML) phylogenetic trees were inferred from the sequence alignments by using PhyML version 3.0 (*15*). Phylogenies inferred from nucleotide and amino acid sequences used the general-time reversible and Whelan-and-Goldman substitution models, respectively. A discrete-γ distribution of 4 rate categories (Γ_4) was used to model among-site heterogeneity. The robustness of the tree topology was assessed by bootstrap analysis of 1,000 pseudo-replicates of the sequences.

Molecular Clock Dating

The evolutionary time scale of 2 novel human betaCoVs, EMC/2012 and England/Qatar/2012, was estimated by using a strict clock model under the Bayesian Markov Chain Monte Carlo framework in BEAST version 1.7.4 (*16*). The Hasegawa-Kishino-Yano nucleotide substitution model was used with a discrete-γ distribution of 4 rate categories (Γ_4) to enable among-site heterogeneity. A simple constant coalescent prior on the age of the divergence was used. With only 2 samples, estimating a rate of evolution was not possible, so we conditioned our date estimates on a range of fixed rates from 1.0×10^{-4} to 5.0×10^{-3} substitutions/site/year to enable comparison with plausible values. For each fixed rate value, a total of 10^7 Bayesian Markov Chain Monte Carlo states were computed, with the first 10% discarded as burn-in.

Results

Comparison of England1, England/Qatar/2012, and EMC/2012

The index genome for this virus, EMC/2012, was originally obtained from a Saudi Arabian patient in July 2012 after 6 passages in cell culture (*3*). Our England/Qatar/2012 sequence was derived directly from the sputum of a Qatari patient receiving care in London in September 2012. A consensus genome from the same Qatari patient, but from a lower respiratory tract sample obtained later in the infection, is also available (England1, GenBank accession no. KC164505). The 3 genomes were aligned to determine sequence differences.

The England/Qatar/2012 and England1 genomes show only nucleotide differences at the genome termini, with the England1 genome lacking 46 nt at the 5′ end and 42 nt at the 3′ end (Figure 2, panel A, Appendix, wwwnc.cdc.gov/EID/article/19/5/13-0057-F2.htm). The England1 genome was generated by using 80 PCR products sequenced by Sanger dideoxy methods (*17*), and the high level of sequence identity between the England/Qatar/2012 and England1 sequences strongly validates the novel rapid sequencing methods described here.

The England/Qatar/2012 consensus genome has 30,021 (99.67%) of 30,119 nucleotides identical to the EMC/2012 genome. In addition to the single nucleotide differences, EMC/2012 shows an insertion of 6 nt starting at position 29639 and a single A insertion at 30661 relative to the England/Qatar/2012 genome. The sequence differences are evenly spread across the genome (Figure 2, panel A, Appendix; Table 1).

Deep sequencing data enable the generation of a consensus sequence from the majority nucleotide at each genome position and the identification of nonconsensus nucleotides at each position. The England/Qatar/2012 sample was sequenced to a high level of coverage (mean coverage = 4,444) compared with the previously reported England1 genome (2–11-fold), enabling insights into the variation consequent on within-host evolution. Setting a conservative estimate for total sequencing errors at 1% enables nucleotide variants present at >1% frequency to be considered true variants in the virus genome (Figure 2, panel B, Appendix). Variation is clearly detected across the virus genome with certain regions, such as nonstructural protein (NSP) NSP3, NSP5, NSP6, NSP12, and NSP14 showing increased levels of nonconsensus nucleotides (Table 2).

The predicted ORFs in England/Qatar/2012 are shown in Figure 2, panel C, Appendix. The ORF pattern is identical to EMC/2012, with 1 exception: England/Qatar/2012 has a G at position 27162 and a longer ORF 5, whereas EMC/2012 has an A (and the predicted ORF 5 is truncated). Van Boheemen et al. (*3*) had commented on mixed nucleotides (A–G) observed at this position. In addition, EMC/2012 had variation at position 11623 (U or G), whereas the England/Qatar/2012 genome has a U at this position.

An assessment of specific ORFs of the 2 viral genomes was performed because evolution of specific proteins is a key determinant of host range and defines cross-species transmission events that may be recent for this virus. As expected from the overall close homology of the 2 viruses,

several ORFs show little or no change between the 2 genomes (Table 1). However, several ORFs show higher amino acid differences, including the nucleocapsid ORF N, and ORFs 3, 4a, and 8b (Table 1).

Of the 16 predicted nonstructural proteins encoded by ORF 1a and ORF 1b, 11 show no change at the amino acid level (Table 1), whereas 5 (NSP2, NSP3, NSP4, NSP13, and NSP15) show >0.3% difference (Table 1). As more sequences become available from this virus, such comparisons will yield clues about the adaptation to humans.

Close Phylogenetic Relationship with European Bat CoVs

A ML phylogenetic tree inferred from the whole genome alignment indicated that the 3 novel human betaCoVs sequences (England1, England/Qatar/2012, and EMC/2012) clustered closely, forming a monophyletic lineage that falls into group 2c (Figure 3, panel A, Appendix, wwwnc.cdc.gov/EID/article/19/5/13-0057-F3.htm). This novel human betaCoV lineage shares common ancestries with other group 2c bat CoV variants, including HKU4 and HKU5 strains isolated in southern People's Republic of China (Figure 3, panel A, Appendix); however, the genetic distances between them remain substantial (≈70% similarity in nucleotide level). The novel human betaCoVs have even less similarity with group 2a, 2b, and 2d CoVs (<60% nt). Phylogenies constructed from individual ORFs including ORF 1ab, S, E, M, and N demonstrated a largely consistent phylogenetic position of the novel human betaCoVs, except for a small discordance in the order of branching between novel human betaCoV, HKU4, and HKU5 lineages (Figure 3, panels B–F, Appendix). Such phylogenetic incongruence could result from several possible evolutionary features of the virus, including evolutionary rate variation, homoplasy, and recombination, as well as from uncertainty in the alignment of distant sequences. Additional related sequences are needed to clarify this issue.

Table 1. Nucleotide and amino acid differences between novel human betacoronaviruses EMC/2012 and England/Qatar/2012 major ORFs

	Nucleotide		Amino acid	
ORF*	Difference†	Change, %‡	Difference†	Change, %‡§
ORF 3	4	1.28	2	1.92
N	11	0.86	4	0.94
ORF 8b	2	0.59	1	0.88
ORF 4a	3	0.88	1	0.88
NSP13	4	0.24	4	0.71
NSP2	7	0.35	4	0.61
NSP15	3	0.29	2	0.58
ORF 5	3	0.44	1	0.44
NSP3	21	0.37	8	0.42
NSP4	3	0.20	2	0.39
ORF 4b	4	0.46	1	0.34
ORF 1a	45	0.34	14	0.32
S	10	0.24	2	0.15
ORF 1b	15	0.19	3	0.11
E	0	0.00	0	0.00
M	0	0.00	0	0.00
NSP1	2	0.35	0	0.00
NSP5	2	0.22	0	0.00
NSP6	4	0.46	0	0.00
NSP7	1	0.40	0	0.00
NSP8	1	0.17	0	0.00
NSP9	3	0.91	0	0.00
NSP10	1	0.24	0	0.00
NSP11	0	0.00	0	0.00
NSP12	4	0.14	0	0.00
NSP14	3	0.19	0	0.00
NSP16	1	0.11	0	0.00

*ORF nomenclature is from van Boheemen et al. (*3*). ORF, open reading frame; NSP, nonstructural protein.
†No. nucleotide or amino acid differences between aligned ORF or their predicted protein products.
‡No. differences (nucleotide or amino acid) divided by the length of the ORF or the predicted protein.
§ORFs were sorted by decreasing amino acid percentage change.

Because more CoV strains are available in GenBank for partial genomic sequences, we performed extensive BLAST searches (www.ncbi.nlm.nih.gov/blast) and identified several additional European bat CoV sequences sharing higher nucleotide sequence similarity (82.0%–87.7%) with the novel human betaCoVs. Because only partial *RdRp* sequences were available for these European bat CoVs, another ML phylogeny was constructed from this short region to examine their evolutionary relationships (Figure 3, panel G, Appendix). As noted, P.pipi/VM314/2008/NLD (GenBank accession no. GQ259977) identified in a *Pipistrellus pipistrellus* bat from the Netherlands (*18*) shows the closest sequence similarity (87.7%) and phylogenetic relationship to the novel human betaCoVs. In addition, 2 CoV sequences, H.sav/J/Spain/2007 (GenBank accession no. HQ184059; from *Hypsugo savii*, also known as *P. savii*) and E.isa/M/Spain/2007 (HQ184062; from *Eptesicus isabellinus*), obtained from bats from Spain (*19*) are also closely related to the novel human betaCoVs (Figure 3, panel G, Appendix).

Timing the Origin of Zoonosis

The time to the most recent common ancestor (tMRCA) of EMC/2012 (isolated June 13, 2012) and England/Qatar/2012 (isolated September 19, 2012) was estimated with a strict molecular clock model. A plot of estimated tMRCA of the 2 human strains as a function of the fixed rate of molecular evolution shows that with the fastest measured rate of human CoVs (2×10^{-3} substitutions/site/year for SARS-CoV [*20*]) the tMRCA (i.e., October 2011; 95% highest posterior density: August–December 2011) is older than the start of 2012, but with slower rates it is calculated to be much older (Figure 4 [*20*,*21*–*23*]). However, for evolutionary rates as high as those of human influenza A virus and HIV (e.g., $3–5 \times 10^{-3}$ substitutions/site/year), the estimated tMRCA becomes compatible with the earliest disease report in Jordan in April 2012 (*7*).

Discussion

The reports of several human infections by similar strains of a novel betaCoV have raised global concern about a new SARS-like outbreak. Such worry is not misplaced, particularly when little is known about the origin and transmissibility of the virus and the natural history of the disease it causes. In this study, we presented a rapid deep sequencing method to obtain the complete genome sequence of the novel human betaCoV and its minor variants from a patient's sputum sample, which provides a useful tool to study the origin, evolution, and detection of this novel CoV. Our detailed phylogenetic analyses of the viral genomes also provide additional clues to the emergence and evolution of this virus.

Mammalian zoonoses are often identified as the origin of new human CoV infections including OC43, NL63, and SARS-CoVs (*24*–*27*). In particular, bats, which maintain the largest diversity of CoVs (*28*), are thought to be a natural reservoir of the virus and thus a probable origin of the novel human betaCoV studied here. Indeed, the initial genetic study of the novel human betaCoV demonstrated its phylogenetic relatedness to the HKU5 and HKU4 bat CoVs in southern China (*5*). van Boheemen et al. found a bat CoV sequence from the Netherlands that clusters closely with EMC/2012 (*3*). In addition, most bat CoVs in the group 2c lineage, including the sequence from the Netherlands, were isolated from bat species of the Vespertilionidae family, whereas the SARS-like CoVs in group 2b lineage were mainly from bats from the Rhinolophidae family (*29*). In this study, we showed that 2 other CoVs from bats from Spain of Vespertilionidae again are also clustered near the novel human betaCoVs (Figure 3, panel G, Appendix), which further supports a European Vespertilionidae bat ancestry for this virus. However, such a genealogic link may be indirect and distant because a similar hypothesis for SARS-CoV has been under dispute: whether the CoV jumped from bats directly to humans or through civets or even through some other animals as intermediate hosts (*30*). Therefore, like SARS-CoV, the high dissimilarity between the bat CoVs and novel human betaCoVs makes it difficult to rule out the presence of other intermediate hosts transferring the virus from bats to humans and to confirm the geographic origin of the direct predecessor. With the current data, Europe and the Middle East would be plausible regions for more intensive pilot surveillance studies on bats and other animal reservoirs for this virus.

In the interest of public health, it is critical to determine whether these CoV infections in humans are the consequence of a single zoonotic event followed by ongoing human-to-human transmissions or whether the 3 geographic sites of infection (Jordan, Saudi Arabia, and Qatar) represent independent transmissions from a common

Table 2. Nonconsensus variants detected in the sequence of a novel human betacoronavirus

Name*	Start†	End	≥0.01–0.05‡	>0.05–0.1	>0.1	Total§	Length	%¶#
NSP9	12659	12988	3	0	0	3	329	0.91
NSP10	12989	13408	3	0	0	3	419	0.72
NSP14	18001	19572	6	1	1	8	1571	0.51
NSP6	10937	11812	4	0	0	4	875	0.46
NSP3	2837	8497	25	0	0	25	5660	0.44
NSP12	13432	16206	11	1	0	12	2774	0.43
NSP7	11813	12061	1	0	0	1	248	0.40
NSP5	10019	10936	3	0	0	3	917	0.33
N	28565	29806	4	0	0	4	1241	0.32
S	21455	25516	12	0	1	13	4061	0.32
ORF 4a	25851	26180	1	0	0	1	329	0.30
M	27852	28511	2	0	0	2	659	0.30
ORF 8b	28761	29099	1	0	0	1	338	0.30
NSP13	16307	18000	5	0	0	5	1693	0.30
NSP4	8498	10018	4	0	0	4	1520	0.26
NSP2	857	2836	4	0	0	4	1979	0.20
NSP8	12062	12658	1	0	0	1	596	0.17
ORF 4b	26092	26832	0	1	0	1	740	0.14
NSP15	19573	20601	1	0	0	1	1028	0.10
NSP1	278	856	0	0	0	0	578	0.00
NSP11	13409	13453	0	0	0	0	44	0.00
NSP16	20602	21513	0	0	0	0	911	0.00
ORF 3	25531	25842	0	0	0	0	311	0.00
ORF 5	26839	27513	0	0	0	0	674	0.00
E	27589	27837	0	0	0	0	248	0.00

*ORF nomenclature is from van Boheemen et al. (*3*). ORF, open reading frame; NSP, nonstructural protein.
†Nucleotide position in England/Qatar/2012 genome.
‡No. nucleotide positions showing the indicated fraction of nonconsensus nucleotides. Only positions with a minimum of 1,000-fold coverage and a Phred quality score of 30 were included.
§No. nucleotide positions with ≥0.01 (1%) nonconsensus nucleotides.
¶Total no. positions with variation divided by length of ORF) × 100.
#ORFs were sorted by decreasing amino acid percentage change.

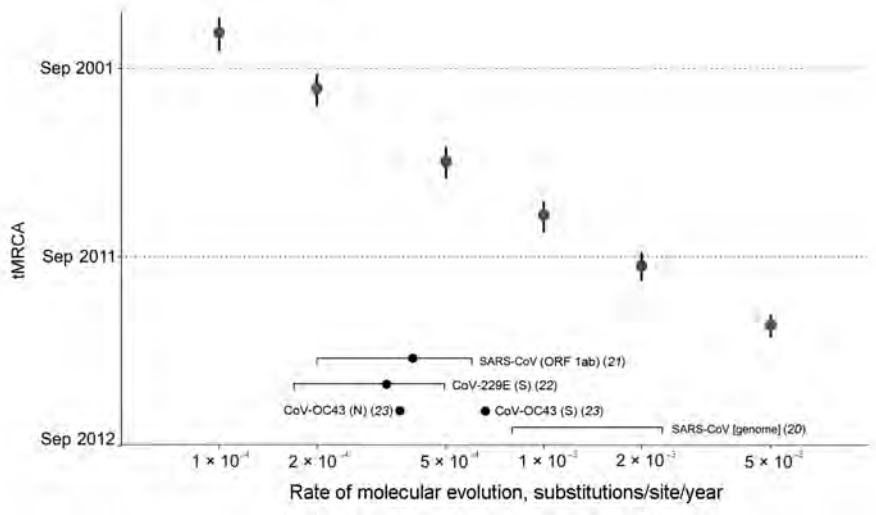

Figure 4. tMRCA analysis across a range of fixed evolutionary rates. The tMRCA of EMC/2012 and England/Qatar/2012 estimated from fixing a range of genomic evolutionary rates (1×10^{-4}, 2×10^{-4}, 5×10^{-4}, 1×10^{-3}, 2×10^{-3}, and 5×10^{-3} substitutions/site/year) are shown in data points with vertical error bars (95% highest posterior density). Evolutionary rate estimates of human CoV genome and genes in the literature are indicated at the bottom of the plot (mean or point estimate as a dot, 95% CIs of estimate as a square bracket). tMRCA, time to the most recent common ancestor; CoV, coronavirus; SARS, severe acute respiratory syndrome; ORF, open reading frame.

nonhuman reservoir. The large genetic diversity of CoV maintained in animal reservoirs suggests that viruses that independently moved to humans from animals at different times and places are likely to be reasonably dissimilar in their genomes, possibly making the multiple transmission events model less likely. Further information is needed to confirm this point because the currently available data are limited. If we calibrate our molecular clock analysis using the evolutionary rate of Zhao et al. (*20*) estimated for SARS-CoV, we dated the tMRCA of EMC/2012 and England/Qatar/2012 viruses to early 2011. Therefore, if both sequenced viruses and the other cases descended from a single zoonotic event, then this tMRCA suggests that the novel virus has been circulating in human population for >1 year without detection and would suggest most infections were mild or asymptomatic. The rate would have to be considerably faster, of a magnitude observed for human influenza A virus, for the tMRCA to be compatible with the earliest known cases in April 2012. Perhaps more probable, therefore, is that the 13 known cases of this disease represent >1 independent zoonotic transmission from an unknown source. Viral sequence data from other patients infected with this novel human betaCoV will help to more accurately estimate the estimate a genomic evolutionary rate specific to this virus, which will then yield a tMRCA estimate closer to the actual time.

A major determinant of SARS pathology is the distribution of the host receptor that the virus has evolved to use for entry. A detailed analysis of EMC/2012 receptor use in cell culture (*31*) revealed that EMC/2012 was capable of infecting a range of mammalian cells, including human, pig, and bat cells, and the virus entry was independent of the ACE2 receptor. Comparing the S genes of England/Qatar/2012 predicts only 2 aa changes (in a 1,367-residue protein). Thus, this broad infection capability of EMC/2012 is likely to be valid for England/Qatar/2012 and would imply a higher possibility for the existence of other intermediate hosts transferring the virus from bats to humans.

Precise identification of the origin of this virus, defining its mode of evolution, and determining the mechanisms of viral pathogenesis will require full-genome sequences from all cases of human infection and substantially more sampling and sequencing from Vespertilionidae bats and other related animals. The sequencing method reported here markedly shortens the time required to process the clinical sample to genome assembly to 1 week and will provide a useful tool to study this novel virus.

Note added in proof. The current number of confirmed novel CoV cases is 17, including 11 deaths (www.who.int/csr/don/2013_02_21/en/index.html). An analysis of the 4 novel CoV genomes now available suggests an evolution rate of 1.77×10^{-3} substitutions/site/year, which would give a tMRCA of mid-2011. The analysis is online (http://epidemic.bio.ed.ac.uk/ coronavirus_analysis).

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Targeting Surveillance for Zoonotic Virus Discovery

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We analyzed a database of mammal–virus associations to ask whether surveillance targeting diseased animals is the best strategy to identify potentially zoonotic pathogens. Although a mixed healthy and diseased animal surveillance strategy is generally best, surveillance of apparently healthy animals would likely maximize zoonotic virus discovery potential for bats and rodents.

Nearly two thirds of emerging infectious diseases that affect humans are zoonotic, and three fourths of these originate in wildlife, making surveillance of wildlife for novel pathogens part of a logical strategy to prevent the future emergence of zoonoses (*1*–*4*). Wildlife are thought to harbor a high diversity of unknown pathogens, but global characterization of this diversity would be costly and logistically challenging (*5*). Given limited resources for pandemic prevention, there is public health benefit in focusing pathogen discovery on those species most likely to harbor novel zoonoses (*3*,*4*).

One strategy to maximize the likelihood of discovering novel pathogens is surveillance of animal die-offs, outbreaks in wildlife, or diseased wildlife. We analyzed a database of known zoonotic viruses in mammal hosts to answer the driving question of whether we should stratify surveillance strategies (i.e., conduct surveillance of visibly diseased vs. apparently healthy animals) by wildlife host groups to best detect novel pathogens with zoonotic potential. In answering this question, we can better determine how host and virus taxonomy might influence our decisions about applying limited surveillance resources to a growing global health problem.

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Methods

We focused our analysis on mammalian hosts and viruses because they, more than any other host–pathogen type, are likely to be associated with emerging infectious diseases of humans (*3*,*6*). We constructed a database of all emerging viruses of humans that were previously identified as originating in wildlife; the database was supplemented with all zoonotic viruses with nonhuman mammalian hosts found in the International Committee on the Taxonomy of Viruses database (www.ictvdb.org) (*2*). For each zoonotic virus, we conducted a literature search for reports of infection in any mammalian host, using the virus name and relevant synonyms (www.ictvdb.org) as keywords in Web of Knowledge (http://wokinfo.com/), Wildlife Disease Association meeting abstracts (http://wildlifedisease.org/wda/CONFERENCES.aspx), Google Scholar (http://scholar.google.com/), and the Global Mammal Parasites Database (www.mammalparasites.org). The resulting 605 host–pathogen relationships included 56 unique viruses classified in 17 taxonomic families and 325 unique mammals classified in 15 taxonomic orders. We excluded rabies from our analysis because the intense research effort on this virus and its high pathogenicity in almost all of its wide range of hosts (*7*) would skew the data disproportionately.

We then conducted a secondary literature search to determine whether viruses in our database cause signs of disease in their wildlife hosts. For the search, we used an aggregate of all publications available in PubMed (www.ncbi.nlm.nih.gov/pubmed/), Web of Science (http://thomsonreuters.com/products_services/science/science_products/a-z/web_of_science/), BIOSIS Previews (http://thomsonreuters.com/products_services/science/science_products/a-z/biosis_previews/), and Biologic & Agricultural Index Plus (www.ebscohost.com/academic/biological-agricultural-index-plus); search terms consisted of virus names and International Committee on the Taxonomy of Viruses synonyms, host genus and species names,

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and common names [reconciled to the 2005 version of Mammal Species of the World (*8*)]. All resulting abstracts and available full text reports were examined until the first robust report of visible disease was encountered. Viruses were identified as causing visible disease in a host if individual or epizootic death or grossly visible or otherwise observable signs of illness (e.g., high fever, loss of mobility, or severe decline in body condition) were reported. A report was considered robust only if infections were confirmed by PCR analysis or virus isolation and clinical signs were explicitly recorded to have occurred during active infection. We excluded studies reporting only serologic findings because of potential cross-reactivity among related viruses and poor correlation between serologic status and concurrent infection. Our criteria of stopping a search once any evidence for visible disease was found meant that for mammal–virus pairs without visible disease, the search was exhaustive.

We considered diseases to be nonpathogenic in their hosts only if actively infected animals were explicitly reported to be free of visible disease. Animals with less clear signs of disease, such as nasal discharge or death of neonates, were not considered asymptomatic because of the low detection probability associated with these traits in wild mammal surveillance. We rejected reports of experimentally induced disease because of the risk that dosage and inoculation technique would not be consistent with naturally occurring infections. However, we included experimental studies if actively infected animals remained asymptomatic, with the assumption that 1) clinical signs of infection were most likely to be seen in animals monitored in laboratory settings than in the wild and 2) stressful conditions in captivity would heighten the likelihood of a normally benign pathogen leading to clinical signs (*9*). Furthermore, compared with naturally occurring infections, experimental infections often involve more direct routes of inoculation and are therefore more likely to induce disease.

Analyses

We conducted a logistic regression analysis, using Firth's bias reduction procedure (*10*) as used by the brglm (bias reduction in generalized linear models) package of R v2.15-2 (http://cran.r-project.org/bin/windows/base/), of apparent host disease as a function of host taxonomy and virus taxonomy for the subset of mammal–virus pairs for which the host order or virus family had at least 3 records in the database. We then calculated odds ratios for each host taxonomic order and virus family relative to the reference categories (Artiodactyla and *Flaviviridae*) and the predicted probability of being symptomatic for all species order–virus family combinations.

Results

Our search of the 605 mammal–virus associations investigated yielded explicit information on host health in 52% of the 312 mammal–virus pairs. Of these, ≈28% (n = 88) of infected wildlife hosts were reported to have had visible disease and 72% (n = 224) were reported without evidence of visible disease (Figure 1, panel A). The proportion of hosts that were symptomatic differed across host order (Figure 1, panel B) and virus family (Figure 1, panel C).

We found that virus family and host order were significant predictors of disease status ($\chi^2 = 88.70$, $p<0.001$ and $\chi^2 = 59.45$, $p<0.001$, respectively). Species infected with paramyxoviruses, poxviruses, and reoviruses were more likely to have visible disease ($p = 0.02$, $p = 0.001$, and $p =$

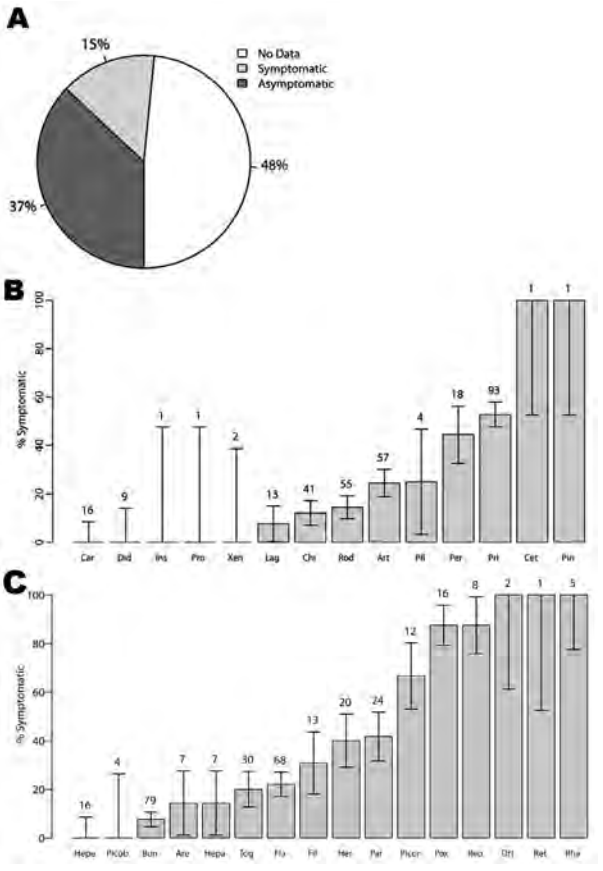

Figure 1. A) Percentage of host–virus pair reports describing symptomatic (observable) disease, asymptomatic disease (no observable disease), or no data (no description of disease included). B) Percentage of symptomatic hosts by mammal taxonomic order. C) Percentage of viruses, by taxonomic family, for which hosts are reported symptomatic. SEs (error bars) were calculated assuming binomial error structure. The total number of each host order or virus family included in the database is given above each bar. All host orders and virus families in the database are included here, but analyses are limited to those host orders or virus families with at least 3 entries in the database. See the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/5/12-1042-Techapp1.xlsx) for the full database of host–virus pairs and disease states.

Table. Logistic regression analysis with bias reduction of whether a host presents with disease for 234 mammal–virus pairs from 5 taxonomic orders of mammals and 10 taxonomic families of viruses*

	Values for categorical predictors relative to level of reference category					
Predictor†	Coefficient	SE	Z test statistic	p value	Odds ratio	95% CI
Constant	–0.33	0.58	–0.56	0.58	0.72	0.23–2.26
Virus family (reference category: *Flaviviridae*)						
Bunyaviridae	–1.74	0.64	–2.71	0.01	0.18	0.05–0.62
Filoviridae	3.26	1.83	1.78	0.08	26.07	0.72–944.49
Herpesviridae	0.10	0.65	0.16	0.87	1.11	0.31–3.94
Paramyxoviridae	3.43	1.42	2.41	0.02	30.95	1.90–503.52
Picornaviridae	1.12	0.76	1.48	0.14	3.08	0.69–13.68
Poxviridae	2.29	0.81	2.82	<0.001	9.90	2.01–48.72
Reoviridae	2.13	1.05	2.02	0.04	8.39	1.07–66.12
Rhabdoviridae	9.20	2.39	3.85	<0.001	‡	‡
Togaviridae	–0.36	0.63	–0.58	0.56	0.70	0.20–2.38
Species order (reference category: Artiodactyla)						
Chiroptera	–6.47	1.81	–3.57	<0.001	0.00	0–0.05
Perissodactyla	0.58	0.76	0.77	0.44	1.79	0.40–8.03
Primates	–0.16	0.68	–0.24	0.81	0.85	0.22–3.24
Rodentia	–1.12	0.67	–1.66	0.10	0.33	0.09–1.22

*The subset of data used was selected by using a cutoff of at least 3 records in the database to avoid making inference about host orders or virus families, for which we had very little information.
†Virus and host reference groups were selected as those for which sample size was sufficiently large and symptomatic infection was moderate (see Figure 1).
‡All host–virus pairs were symptomatic.

0.04, respectively), and species infected with bunyaviruses were less likely to have visible disease relative to the reference category (p = 0.01) (Table). Hosts infected with filoviruses were marginally more likely to have visible disease (p = 0.08) (Table).

Relative to the reference category, species classified in the order Chiroptera were less likely to have visible disease (p<0.001), and species in the order Rodentia were marginally less likely to have visible disease (p = 0.10) (Table). Compared with species in other orders, species in the order Chiroptera had a lower probability of visible disease (Figure 2), although all Chiroptera species infected with nonrabies rhabdoviruses had a high probability of visible disease. In the dataset, all host–pairs infected with rhabdoviruses were in the order Chiroptera and were reported with visible disease in that host (Figure 1).

Discussion

Nonhuman primates (*11–13*) and species classified within the taxonomic orders Chiroptera and Rodentia are the primary mammals targeted for zoonotic disease surveillance. Our data suggest that species in the orders Chiroptera and Rodentia are less likely than species in other orders to have visible disease (Figure 1). The mechanism behind this relationship is a critical area for additional research. In general, we found that the probability of having visible disease depends on the taxonomic classification of the host and virus, and Chiroptera is the only host order for which a single strategy (in this case, healthy animal surveillance) can be applied across nearly all virus families, excluding *Rhabdoviridae*. Therefore, particularly for the case of novel virus detection, our results point to a mixed strategy of targeted syndromic and healthy animal surveillance across host and virus taxonomies. A mixed strategy could combine apparently healthy animal surveillance (particularly in Chiroptera) with syndromic surveillance in other wildlife and domestic animal hosts. Syndromic surveillance has proven useful where secondary animal hosts are involved [e.g., surveillance for West Nile virus (*14*), henipaviruses (*15*,*16*), and Ebola virus (*17*)].

There are limitations to our study, particularly ascertainment and reporting biases, as acknowledged in previous studies of emerging infectious diseases (*2*,*3*). In addition, differences in the number of species belonging to each order, the difficulty of testing inaccessible species, and limits to reliable diagnoses of emerging viruses have an effect, especially in resource-poor settings. Furthermore, many disease states are not recognizable in free-ranging mammalian species under field conditions. Last, there is a risk that an animal may be co-infected with several agents, only one of which causes disease; that co-infection may have an additive or synergistic effect on clinical signs; and that anthropozoonotic viruses artificially inflate the disease count of mammals in some taxonomic orders over others. However, our findings were determined on the basis of an aggregation of the best data available on host health as it relates to zoonotic viruses, and they have useful implications for public health.

Our analysis supports a holistic, probability-based approach to zoonotic virus discovery, specifically, continued analysis of passively and actively reported deaths and increased investment in broad surveillance of healthy wildlife. The latter could be targeted geographically to those regions most likely to generate novel emerging infectious diseases (*2*) or taxonomically to groups that are reservoirs for the highest proportion of zoonoses (*3*,*18*). These efforts

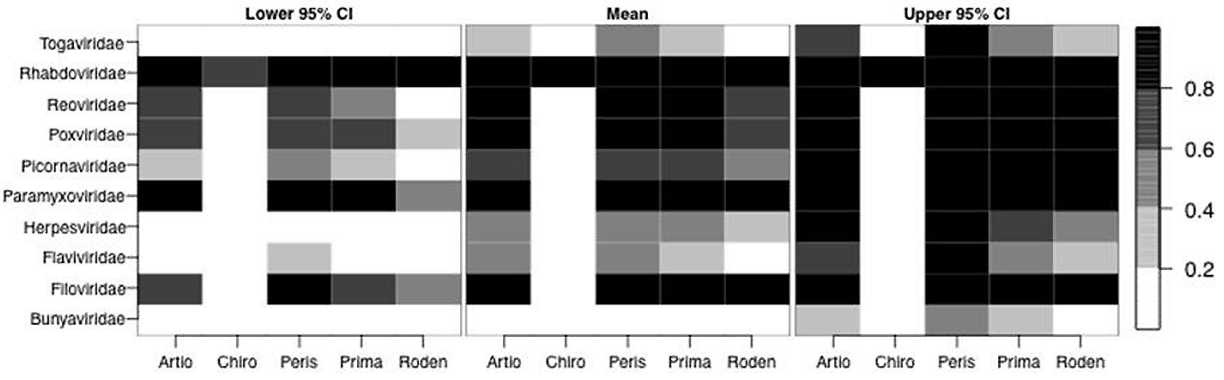

Figure 2. Probability of being symptomatic as determined by logistic regression analysis, with bias reduction of whether a host is diseased, for 234 mammal–virus pairs. Pairs represent mammals from 5 taxonomic orders and viruses from 10 taxonomic families. Probabilities are based on the predicted values of the logistic regression and are given on a 5-point gray scale (key on right). CIs were calculated as the coefficient plus 1.96 ´ SE (from Table). See Table for detailed results of the regression analysis.

could be envisaged as part of a strategy for smart surveillance, heightening the opportunity for discovery of novel zoonoses, particularly if wildlife are sampled at key interfaces where contact with human or domestic animals (and thus the opportunity for spillover) is highest.

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Ms Levinson recently completed her MSc in International Community Health at New York University, conducting research on patterns in emerging infectious viruses as a biological research associate at EcoHealth Alliance. She is currently an Urban Agriculture Volunteer in Kolda, Senegal, with Peace Corps Senegal and is interested in international health and development.

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Changes in Severity of Influenza A(H1N1)pdm09 Infection from Pandemic to First Postpandemic Season, Germany

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We studied risk factors for a severe clinical outcome in hospitalized patients with laboratory-confirmed influenza A(H1N1)pdm09 infection at the University Hospital Heidelberg in the pandemic and first postpandemic seasons. We identified 102 patients in 2009–10 and 76 in 2010–11. The proportion of severely diseased patients dramatically increased from 14% in 2009–10 to 46% in 2010–11 as did the mortality rate (5%–12%). Patients in the first postpandemic season were significantly older (38 vs. 18 years) and more frequently had underlying medical conditions (75% vs. 51%). Overall, 50 patients (28%) had a severe clinical outcome, resulting in 14 deaths. Multivariate analysis showed that older male patients with chronic lung disease were at increased risk for a severe clinical outcome. In summary, the proportion of patients with severe disease and fatal cases increased in the postpandemic season. Therefore, patients with suspected infections should be promptly identified and receive early treatment.

Influenza pandemics have been associated with increased illness and death. Each pandemic is different, and areas of uncertainty always exist when an influenza virus emerges and becomes pandemic. In April 2009, the novel influenza A(H1N1)pdm09 virus emerged in Mexico and then spread rapidly throughout the world (*1*). Influenza is generally a self-limiting infection with systemic and respiratory symptoms that usually resolve after 3–6 days. Most persons infected with the 2009 influenza A(H1N1)pdm09 virus experienced uncomplicated illness with full recovery within 1 week, even without medical treatment; severe progressive disease developed in only a small subset of patients (*2*). Primary viral pneumonia was the most common finding in severe cases, but secondary bacterial infections played a role in ≈30% of fatal cases (*3*). Hospitalized patients were often affected by other medical conditions, such as diabetes and cardiovascular, neurologic and pulmonary diseases (*4*). Advances in therapy for malignancies, autoimmune disorders, and end-stage organ diseases have led to improved survival, but also to an increase in the number of immunosuppressed patients. These patients are particularly at risk for opportunistic and community-acquired infections, such as respiratory virus infections, resulting in considerable illness and death (*5*).

Although patients hospitalized with pandemic influenza A(H1N1)pdm09 infection had substantial severe illness, the overall number of deaths was lower than reported in the earliest studies. The overall number of deaths caused by influenza A(H1N1)pdm09 infection was similar to that caused by seasonal influenza and lower than that of previous pandemics (*6*). The most common cause of death was respiratory failure (*7*). Other reported causes of death included pneumonia, high fever leading to neurologic sequelae, dehydration from excessive vomiting and diarrhea, and electrolyte imbalance. Severe cases were most frequent in middle-aged patients, who often had coexisting conditions (*7*). Although to date there seems to be no major difference between the virulence of influenza A(H1N1)pdm09 strains and seasonal influenza (*8*) strains, a more aggressive course in specific populations, such as in young patients and pregnant women, has been reported (*9*,*10*). Further risk factors include obesity, chronic lung disease, chronic heart disease, chronic renal disease, diabetes mellitus, and severe immunosuppression (*4*,*11*,*12*). Contradictory findings have been reported in regard to varying disease severity during the pandemic season. Although some researchers did not observe any

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differences in disease severity between the first and second pandemic outbreaks in 2009 (*13*,*14*), another study showed a 4-fold increase in hospitalization and a 5-fold increase in number of deaths in the second wave (*15*). However, disease severity of postpandemic seasons has been rarely analyzed.

We performed a retrospective analysis of all patients with laboratory-confirmed influenza A(H1N1)pdm09 virus infection who were hospitalized at the University Hospital Heidelberg, Germany, in the pandemic season 2009–10 and the first postpandemic season 2010–11 to compare the rates of severely diseased patients in both seasons and to identify possible risk factors associated with severe clinical outcome.

Methods

Patients and Study Site

We conducted a retrospective cohort study of all patients admitted to the University Hospital Heidelberg, Germany, with laboratory-confirmed influenza A(H1N1) pdm09 infection from May 2009 through April 2011. We defined a case-patient as a hospitalized person with influenza-like illness and influenza A(H1N1)pdm09 virus infection confirmed by real-time PCR. We extracted positive influenza results from our laboratory information system and reviewed the patient charts for clinical and laboratory characteristics as described below. Additionally, we differentiated between pandemic and postpandemic season and severe and nonsevere infections. Severe disease was defined as either admission to the intensive care unit (ICU) or in-hospital death. Microbiologic studies, hospital and ICU admission criteria, and treatment decisions were not standardized but made at the discretion of the attending physicians.

Data Collection

We collected data on demographic characteristics, coexisting conditions, clinical signs and symptoms, biochemical analyses, chest radiograph findings, antiviral and antibacterial therapy, concomitant and secondary bacterial infections as well as outcome, including death. Pneumonia was defined as the presence of a new infiltrate shown on a chest radiograph plus fever (temperature >38°C) and respiratory symptoms. Bacterial infections were diagnosed by means of blood cultures and analysis of sputum or bronchoalveolar lavage specimens. Routine laboratory analyses included C-reactive protein level and leukocyte count.

PCR

Nasopharyngeal samples and bronchoalveolar lavage specimens were collected from the patients and either processed within 2 hours or refrigerated at –20°C. PCR-based detection of influenza A(H1N1)pdm09 and seasonal influenza A and B strains was performed by 1-step real-time reverse transcription PCR (*16*). Total RNA from respiratory samples was isolated with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's protocol and reverse transcribed by using random hexamers from the Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany). Reverse transcription PCR analysis was performed with 5.0 μL RNA by using the RNA Virus Master Kit (Roche,) and the LightCycler 480 System (Roche) under the following conditions: 8 min at 50°C; 30 s at 95°C; 50 cycles of 5 s at 95°C, 20 s at 60°C and 1 s at 72°C. The primer pairs were used at a concentration of 10 μM.

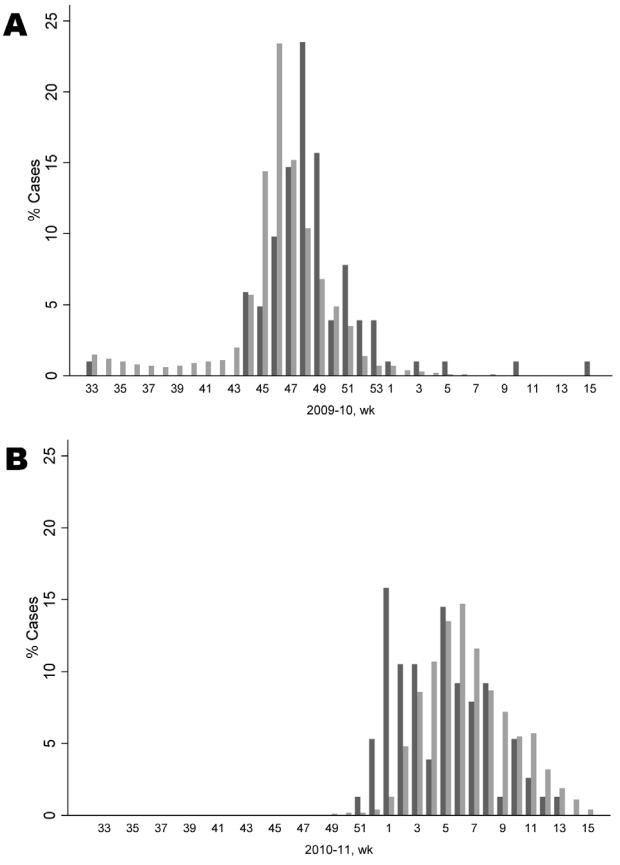

Figure 1. Epidemic curve of influenza A(H1N1)pdm09 virus infections. A) Season 2009–10 and B) season 2010–11. Weeks are indicated; black indicates influenza A(H1N1)pdm09 cases found in the study group at University Hospital Heidelberg; gray indicates influenza cases in Germany.

Statistical Analysis

To describe the temporal distribution of admitted influenza cases, we aggregated cases by calendar week and compared our data with data from the German surveillance

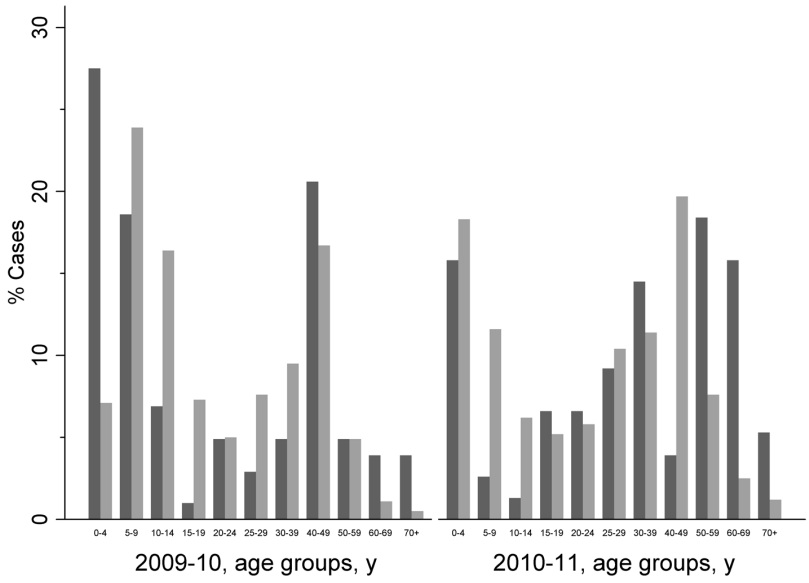

Figure 2. Age distribution of persons with influenza A(H1N1)pdm09 virus infections, winter 2009–10 and winter 2010–11. Black indicates influenza A(H1N1)pdm09 cases found in the study group at University Hospital Heidelberg; gray indicates influenza cases in Germany.

system of mandatory notifiable, laboratory-confirmed influenza cases (*17*). We summarized demographic and clinical data for time and severity. Frequencies were compared by using χ^2 test or Fisher exact test for categorical variables and the Student *t* test or Mann-Whitney U-test for continuous variables, as appropriate. Identified risk factors with a p value <0.2 in the univariate analysis were included in a multivariate logistic regression model to assess independent association with severity. In a stepwise backward procedure, exposures with p>0.05 were excluded from the model. All comparisons with p<0.05 were considered statistically significant. We used Stata version 11 SE (StataCorp. LP, College Station, TX, USA) for all statistical analyses.

Results

Descriptive Epidemiology

We identified 178 hospitalized patients that fulfilled our case definition and included them in the study group.

Table 1. Demographic and clinical characteristics of influenza infected patients stratified by season, 2009–10 and 2010–11, University Hospital Heidelberg, Germany*

Characteristic	No. (%) patients 2009–10	2009–10	p value
Total no. admitted patients	102	76	
Male sex	56 (55)	36 (47)	0.32
Age, mean	18.2	38	<0.05
Days in hospital	7.6	13.9	<0.05
Admitted to ICU	14 (14)	35 (46)	<0.05
Severely diseased	15 (15)	35 (46)	<0.05
Died	5 (5)	9 (12)	0.05
Pregnant	4 (4)	5 (7)	0.50
Pneumonia	29 (28)	33 (43)	<0.05
Mechanical ventilation	11 (11)	24 (32)	<0.05
Underlying medical condition	52 (51)	57 (75)	<0.05
Immunosuppression	22 (22)	41 (54)	<0.05
Cancer	2 (2)	4 (5)	0.23
Blood malignancy	10 (10)	15 (20)	0.06
Solid organ transplant	7 (7)	14 (18)	<0.05
Autoimmune	5 (5)	6 (8)	0.41
Other	2 (2)	3 (4)	0.65
Chronic lung disease	9 (9)	13 (17)	0.10
Cardiovascular disease	25 (25)	33 (43)	<0.05
Renal impairment	9 (9)	20 (26)	<0.05
Diabetes	6 (6)	11 (14)	0.05
Metabolic dysfunction	15 (15)	13 (17)	0.66
Neurologic impairment	15 (15)	8 (11)	0.41
CRP level on admission, mg/L†	34.7 (range <2.0–213.6)	69.2 (range <2.0–381.4)	<0.05
Leukocyte count on admission, /nL†	9.9 [range 0.1–108.7)	9.1 (range 0.9–48.8)	

*ICU, intensive care unit; CRP; C-reactive protein.
†Or the following day if not tested on admission.

Table 2. Demographic and clinical characteristics of influenza- infected patients stratified by severity, University Hospital Heidelberg, 2009–10 and 2010–11, Germany*

Characteristic	No. (%) patients		p value
	Nonsevere diseases	Severe disease	
Total no. admitted patients	128	50	
Male sex	61 (48)	31 (62)	0.08
Age, y, mean	18.4	47.9	<0.05
Days in hospital	6.4	20.2	<0.05
Pregnant	8 (6)	1 (2)	0.45
Pneumonia	22 (17)	40 (80)	<0.05
Underlying medical condition	64 (50)	45 (90)	<0.05
Immunosuppression	35 (27)	28 (56)	<0.05
Cancer	1 (1)	5 (10)	<0.05
Blood malignancy	12 (9)	13 (26)	<0.05
Solid organ transplant	16 (13)	5 (10)	0.64
Autoimmune	6 (5)	5 (10)	0.19
Other	2 (2)	3 (6)	0.11
Chronic lung disease	11 (9)	11 (22)	<0.05
Cardiovascular disease	29 (23)	29 (58)	<0.05
Renal impairment	14 (11)	15 (30)	<0.05
Diabetes	5 (4)	12 (24)	<0.05
Metabolic dysfunction	16 (13)	12 (24)	0.06
Neurologic impairment	16 (13)	7 (14)	0.79
CRP level on admission, mg/L†	34.6 (range 1.99–213.6)	86.7 (range 1.99–381.4)	<0.05
Leukocyte count on admission, /nL†	9.4 (range 1.9–108.7)	10.1 (range 0.1–48.8)	0.71

*CRP, C-reactive protein.
†Or the following day if not tested on admission.

In the 2009 influenza pandemic season, August 2009 through April 2010, a total of 102 patients with influenza A(H1N1)pdm09 infection were admitted to the Heidelberg University Hospital. In the first postpandemic season (December 2010–March 2011), the number decreased to 76 patients. During the pandemic season, no influenza cases other than influenza A(H1N1)pdm09 infection were observed, whereas in the first postpandemic season, 4 patients received a diagnosis of influenza B virus infection. However, we did not include these influenza B–infected patients in this study.

Epidemic Curve and Age Distribution of Influenza A (H1N1) Case-Patients

We charted the temporal distribution of influenza case-patients admitted to the University Hospital Heidelberg by date of first positive laboratory result, compared with influenza cases notified to the German national surveillance system by date of notification (Figure 1). The first observed admission occurred in August 2009 when only sporadic cases of influenza were observed in Germany. The number of admissions increased in October during the first wave with measurable effects of disease at the population level, preceding the usual beginning of the seasonal epidemic by 3 months. The distribution patterns of admissions to Heidelberg University Hospital and notified cases in Germany are similar, both in the pandemic and postpandemic year. In the 2010–11 season, the first cases of influenza were diagnosed in late December in Heidelberg, which is consistent with the usual start of the influenza season.

The distribution of influenza cases among age groups is shown in Figure 2. In the pandemic season >50% of cases from the German national surveillance system were in adolescents (5–19 years of age). In the subsequent season, this pattern shifted to younger children with >50% of cases in patients <14 years of age. The age distribution of patients admitted to the University Hospital of Heidelberg differed from the German national data with higher rates of children <10 years of age in 2009–10 and higher rates in persons >50 years of age in the 2010–11 season.

Comparison of the 2 Seasons

The demographic and clinical characteristics of patients by influenza season are compared in Table 1. In both seasons, the sex distribution was balanced (55% male patients in 2009–10 vs. 47% in 2010-11). However, patients in the first postpandemic season tended to be older (mean age 38 vs. 18 years, $p<0.05$) than patients in the pandemic season and had a higher proportion of concomitant medical conditions (75% vs. 51%, $p<0.05$). Furthermore, an increased severity was observed in 2010–11 with a doubled mean duration of hospitalization (13.9 days vs. 7.6 days, $p<0.05$) and a tripled rate of patients with severe disease (46% vs. 14%, $p<0.05$). The mortality rate was 12% in 2010–11 compared with 5% in 2009–10 ($p = 0.05$).

Comparison of Patients with Severe and Nonsevere Disease

Of the 178 patients with influenza A(H1N1)pdm09 infection hospitalized in both seasons, 50 patients (28.1%) had a severe course of infection; of these, 14 died. In

Table 3. Factors independently associated with severe clinical outcome, University Hospital Heidelberg, 2009/10–2010/11, Germany*†

Characteristic	No. patients with severe disease	Total no. patients	Relative risk (95% CI)	p value
Age, y				
0–14	5	85	1 (Ref)	
15–64	33	76	6.61 (2.83-15.42)	<0.01
≥65	12	17	9.55 (3.94-23.13)	<0.01
Season				
2009–10	15	102	1 (Ref)	
2010–11	35	76	1.64 (1.02-2.62)	0.04
Chronic lung disease				
No	39	156	1 (Ref)	
Yes	11	22	1.89 (1.16-3.09)	0.01
Sex				
F (not pregnant)	18	77	1 (Ref)	
F (pregnant)	1	9	0.36 (0.06-2.36)	0.29
M	31	92	1.67 (1.09-2.57)	0.02

*Ref, reference category for risk calculations.
†Variables (listed in Table 1) that did not reach the significance level of 5% are omitted.

univariate analysis, severity of disease was significantly associated with age, presence of pneumonia as well as with a history of an underlying medical condition, in particular, immunosuppression, chronic lung disease, cardiovascular disease, renal impairment, and diabetes (Table 2). In multivariate analysis, 4 independent risk factors for a severe clinical outcome were identified (Table 3): age, male sex, chronic lung disease, and infection in the postpandemic season were significantly associated with severity of disease. The rate of bacterial co-infection increased from 10% to ≈30% in the postpandemic season, but this rate may have been underestimated because microbiologic investigations were not performed routinely for all patients (data not shown).

Demographic and clinical characteristics of influenza patients with a fatal outcome are shown in Table 4. Notably, all 14 case-patients who died had an underlying medical condition; 4 of 14 had multiple myeloma.

Multivariable Analysis of Risk Factors for Disease Severity

Our multivariable logistic regression model identified 4 independent risk factors for severe clinical disease (Table 3). Older, male patients with any chronic lung disease were more affected in the postpandemic season. The other variables mentioned in Table 2 did not reach statistical significance and were therefore not included in our final model.

Discussion

In our study, the rate of severely diseased patients with influenza A(H1N1)pdm09 virus infection increased 3-fold in the first postpandemic season, resulting in an in-hospital mortality rate of 12% compared with 5% in the pandemic season. Also, the length of hospital stay doubled in the postpandemic season, and the need for mechanical ventilation and ICU admission were significantly higher. We identified older age, male sex, any chronic lung disease, and diagnosis in the postpandemic season as independent risk factors for a severe clinical course of disease. Our study was performed in a retrospective manner without standardized data collection. Information in the medical records can be incomplete, especially for the classification for severe outcome and underlying medical conditions. Therefore, no established scoring system like the pneumonia severity index could be used in classifying severe clinical course.

Influenza A(H1N1)pdm09 virus in the 2009–10 season affected mainly children and young adults in Germany (*18*). Most hospitalized case-patients in our study group in 2009–10 were young patients, confirming that children and young adults were the age category mostly affected by the pandemic. The low prevalence of older patients in the pandemic season has been attributed to residual immunity against influenza A(H1N1)pdm09 strains in persons born before 1950 (*19*). Previous studies found that current (*20*,*21*) and past pandemics (*22*) affected mainly younger patients. This finding is in contrast with findings of epidemiologic features of seasonal influenza, for which hospitalizations are more common among persons ≥65 years of age and those <15 years.

The highest increase in seroprevalence between pre- and postpandemic periods was observed among patients 18–29 years of age in Germany (*23*). In the United Kingdom, population-based evaluation of serologic immunity to the pandemic strain after the pandemic season suggested that susceptibility was lowest in age groups <15 years, with substantial remaining susceptibility in the those 15–44 years (*24*). Because the remaining susceptibility in children is limited, the probability of extensive illness in this age group with influenza A(H1N1)pdm09 virus in the 2010–11 season was unlikely in the absence of substantial antigenic change in the pandemic virus. Early in the 2010–11 season, high rates of positivity are most marked in those ages 15 to 44 years, the major group contributing to hospital admissions and death (*25*). This result is in contrast

Table 4. Demographic and clinical characteristics of patients infected with influenza A(H1N1)pdm09 who died, Germany, 2010–11*

Patient no.	Age, y/sex	Underlying condition	Bacterial coinfection	Pneumonia type (per chest radiograph)	Complications (other than pneumonia)	Antiviral therapy	Mechanical ventilation	Length of hospital stay, d
1	28/M	Lliver/kidney transplant	No	Bipulmonary	Renal and liver failure, SIRS	Yes	Yes	13
2	75/F	CHD, COPD, post breast cancer	No	Unilobar	Myocardial infarction	Yes	Yes	37
3	68/M	Multiple myeloma	No	Bipulmonary	Pneumothorax	Yes	Yes, + ECMO	56
4	71/M	Multiple myeloma	No	Bipulmonary		Yes	Yes, + ECMO	19
5	18/F	None	No	No	Myocarditis	No	Yes	2
6	57/F	Multiple myeloma	*Enterococcus faecium*	Bipulmonary	RSV coinfection	Yes	Yes, + NO	12
7	52/F	Multiple myeloma	No	Bipulmonary		Yes	Yes, + NO	17
8	85/F	Parkinson disease, epilepsy	No	Bipulmonary	Myocardial infarction	No	Yes, + NO	4
9	65/F	Esophageal carcinoma	No	Bipulmonary	ARDS	Yes	Yes, + NO, + ECMO	52
10	61/F	PBC, chronic hepatitis B, diabetes, COPD	*Enterococcus cloacae, Achromobacter* sp.	Bipulmonary	Renal and liver failure, sepsis, lung edema	Yes	Yes, + NO	53
11	45/F	Acute leptospirosis	*E. faecium, Leptospira* sp.	Bipulmonary	Renal and liver failure	Yes	Yes	13
12	49/M	Colitis ulcerosa	*Escherichia coli*	Bipulmonary	Perforation of colon, hemorrhagic shock, lung edema	Yes	Yes, + NO, + ECMO	8
13	61/F	CHD, CKD	*Klebsiella pneumoniae*	Bipulmonary	Septic shock	Yes	Yes, + NO	28
14	53/F	Liver cirrhosis	*E. faecium*	Bipulmonary	Renal and liver failure, septic shock, CMV reactivation, aspergillosis	Yes	Yes	43
15	52/M	Multiple myeloma, post allogenic transplant	*E. faecium*	Bipulmonary	GvHD 4, cerebral hemorrhage, CMV reactivation, progressive myeloma	Yes	Yes	46

*SIRS, systemic inflammatory response syndrome; CHD, congestive heart disease; COPD, chronic obstructive pulmonary disease; ECMO, extracorporeal membrane oxygenation; RSV, respiratory syncytial virus; NO, nitric oxide; ARDS, acute respiratory distress syndrome; PBC, primary biliary cirrhosis; CKD, chronic kidney disease; CMV, cytomegalovirus; GvHD, graft versus host disease.

to findings in the 2009–10 pandemic when highest rates of infection in the community were observed in children. The increase in severe courses of infection and requirement for critical care in 2010–11 might reflect the effects of influenza A(H1N1)pdm09 illness in the remaining susceptible adults and risk groups in the population, primarily older patients with coexisting conditions—the historically recognized risk factors for seasonal influenza.

Younger age, chronic coexisting conditions, morbid obesity, and bacterial coinfection have been reported as independent risk factors for severe disease in the pandemic season (*3*,*4*). Of hospitalized patients with severe influenza A(H1N1)pdm09 infection, 15%–20% were immunosuppressed in the pandemic season (*3*,*4*). These data are in line with a rate of 27% of immunosuppressed patients in our cohort. It might be speculated that hematologic malignancy or treatment of hematologic disorders is an independent risk factor for fatal disease. Studies involving patients with pandemic influenza A(H1N1)pdm09 infection found that although older patients had the lowest estimated incidence rate, they also had the highest case-fatality rate (*6*). Delayed hospital admission and delayed antiviral therapy have been associated with an unfavorable outcome in the general population and among solid-organ recipients (*12*). The case-fatality rate increased from 5% to 13% in patients we studied; a lower rate of 3% in the pandemic season had been reported previously (*26*). Bacterial coinfection is common in case-patients with a fatal outcome (*27*–*29*) and has also been observed in our patients who died. Thus, timely administration of antiviral and/or antibacterial drug therapy is indicated in high-risk patients.

The increase of severe and even fatal cases in hospitalized patients with influenza A(H1N1)pdm09 infection in the subsequent season of the pandemic has also been reported by the Novel Influenza A (H1N1) Study Group of the Spanish Network for Research in Infectious Diseases (*30*) in Spain. Severe clinical courses have been described for patients with underlying conditions, pregnant women, and obese patients (*4*,*10*). In 2009, this study group observed similar rates of severe disease (*31*) to our observations, namely, 28% with pneumonia and 15% with severe disease. Molecular characterization of influenza A(H1N1)pdm09 viruses circulating in 2010 revealed only a minor genetic drift compared with early isolates from 2009 (*25*), which most likely cannot be attributed for the change in clinical outcome.

In conclusion, we found notable epidemiologic changes and an increased severity of influenza A(H1N1)pdm09 infections in the first postpandemic influenza season. These findings reinforce the need to identify and protect groups at highest risk for adverse outcomes.

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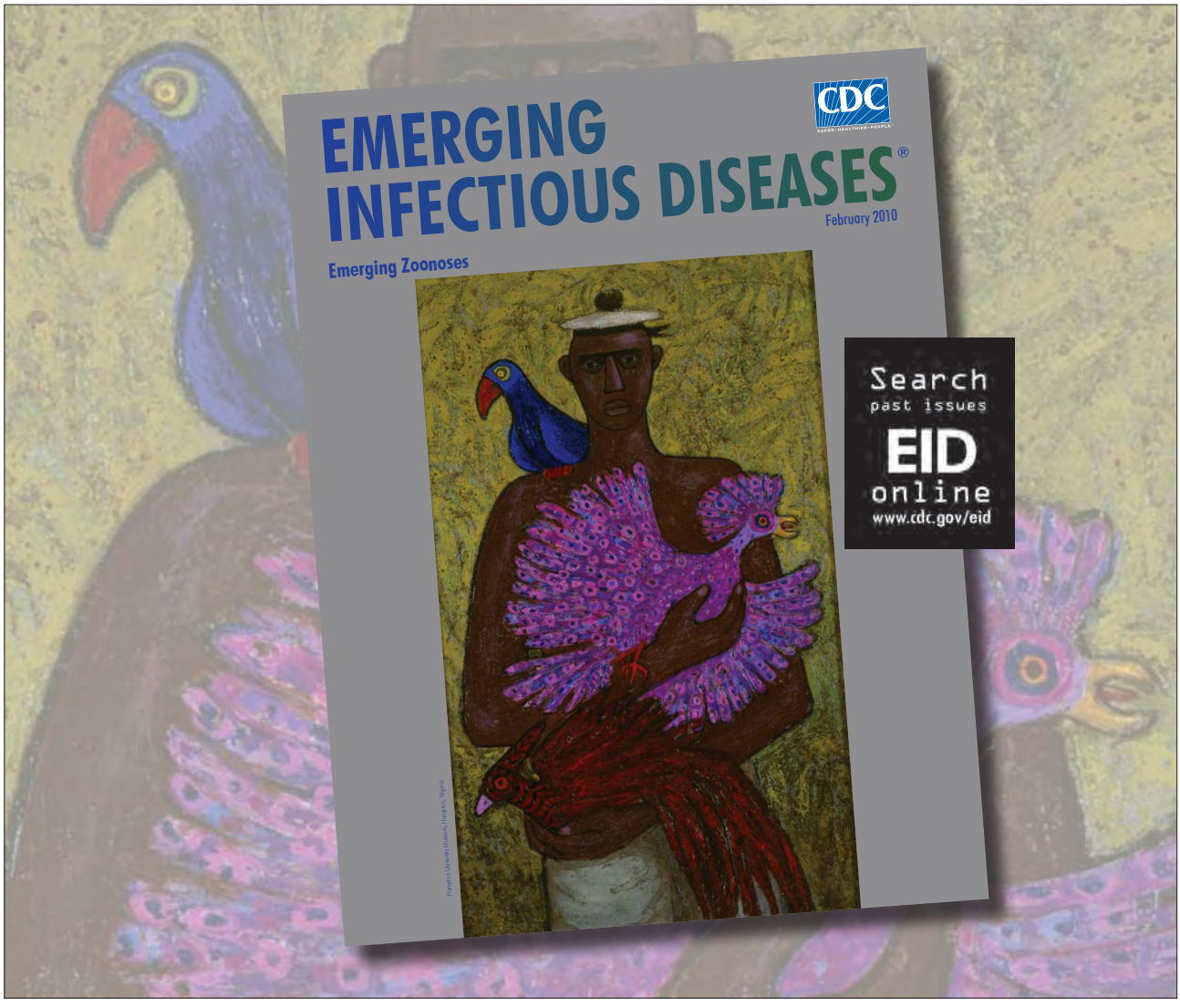

Severe Fever with Thrombocytopenia Syndrome Virus among Domesticated Animals, China

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To investigate the infections of severe fever with thrombocytopenia syndrome virus (SFTSV) in domesticated animals, we sampled a total of 3,039 animals in 2 counties in Shandong Province, People's Republic of China, from April to November 2011. SFTSV-specific antibodies were detected in 328 (69.5%) of 472 sheep, 509 (60.5%) of 842 cattle, 136 (37.9%) of 359 dogs, 26 (3.1%) of 839 pigs, and 250 (47.4%) of 527 chickens. SFTSV RNA was detected in all sampled animal species, but the prevalence was low, ranging from 1.7% to 5.3%. A cohort study in 38 sheep was conducted to determine when seroconversion to SFTSV occured. SFTSVs were isolated from sheep, cattle, and dogs and shared >95% sequence homology with human isolates from the same disease-endemic regions. These findings demonstrate that natural infections of SFTSV occur in several domesticated animal hosts in disease-endemic areas and that the virus has a wide host range.

Severe fever with thrombocytopenia syndrome virus (SFTSV) is a newly identified pathogenic member of the *Phlebovirus* species in the family *Bunyaviridae*, which in humans causes fever with thrombocytopenia syndrome (SFTS) (*1*). The common signs and symptoms of SFTS include high fever, gastrointestinal symptoms, thrombocytopenia, leukocytopenia, and multiorgan dysfunction with an average case-fatality rate of 10%–16%, according to the information system for disease control and prevention, Chinese Center for Disease Control and Prevention (China CDC). In severe SFTS cases, neural symptoms, hemorrhages, disseminated intravascular coagulation, and multiorgan failure can occur and may result in death (*2*).

SFTS has been reported in at least 13 provinces in the central, eastern, and northeastern regions of the People's Republic of China. Most patients are farmers living in wooded, hilly, or mountainous areas, and the epidemic season is from March through November, with the peak incidence usually in June and July (*1*). Although *Haemophysalis longicornis* ticks have been implicated as vectors of SFTSV (*1*,*3*), and high seroprevalence to SFTSV has been reported in goats (*4*,*5*), the host range of the virus has not been determined. The role of domesticated animals in the circulation and transmission of SFTSV remains unclear.

To assess the prevalence of SFTSV infections in domesticated animals, combined cross-sectional and cohort studies were conducted in Laizhou and Penglai in Shandong Province. This study area was selected on the basis of its high incidence of human SFTS cases in 2010 (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/5/12-0245-Techapp1.pdf). We report the detection of viral RNA and antibodies in sheep, cattle, dogs, pigs, and chickens. Our findings may provide valuable insights for understanding SFTSV ecology and transmission among animals and from animals to humans.

Materials and Methods

Study Design and Sample Collection

Animal sampling took place in Laizhou (119°33′–120°18′E, 36°59′–37°28′N) and Penglai (120°35′–121°09′E, 37°25′–37°50′N), Shandong Province, China.

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The most common domesticated animal species in the region include sheep, cattle, dogs, pigs, and chickens (online Technical Appendix). Animals of these species were sampled monthly in the villages where SFTS human cases were reported in 2010. Fifty to 100 animals of each species were sampled each month from April through November of 2011 and the serum samples were collected for detection of viral RNA, antibodies, and virus isolation. Goats were not surveyed because their populations are small in the sampling area.

Each sampled animal was marked with a unique label except for the pigs, which were sampled at the time of slaughter. Sampled animals were excluded in subsequent sampling events. In addition, to monitor new infections of SFTSV, a cohort of 38 sheep (Laizhou, n = 17; Penglai, n = 21) negative for SFTSV viral RNA and antibodies was established, and the animals were sampled every 10 days from June 20 through November 30. Serum samples were aliquoted and kept in cryovials in liquid nitrogen. Viral RNA and antibodies were first screened at the local CDC laboratories in Laizhou and Penglai, respectively, and the results were confirmed later in the national laboratory of China CDC. During the sampling period, serum samples from new SFTS patients in the study region in 2011 were also collected for virus isolation and genetic analysis.

ELISA

Antibodies in sheep, cattle, dogs, pigs, and chickens against SFTSV were detected by using a double-antigen sandwich ELISA described previously (*6*). In brief, a His-tagged affinity chromatography purified recombinant nucleocapsid (N) protein of SFTSV (strain HB29) expressed in *Escherichia coli* was used as the coating antigen for 96-well plates, and horseradish peroxidase (HRP)–conjugated antigen was used for detection. Serum samples obtained from 46 sheep, 55 cattle, 30 dogs, 55 pigs, and 50 chickens originating from either Beijing or Hebei provinces, where no human SFTS cases had been reported, were used as negative controls, and convalescent-phase SFTS patient serum samples were used as positive controls. All serum samples including negative controls were tested at a dilution of 1:10. After 96-well plates were coated with purified N protein (0.2 μg/well), serum samples were added, followed by HRP-conjugated N protein. TMB peroxidase substrate (3,3′,5,5′tetramethylbenzidine and hydrogen peroxide [H_2O_2]) was used for color development, and substrate conversion was measured by using a DTX 880 multimode detector (Beckman Coulter, Brea, CA, USA) with an incidence wavelength of 450 nm and a reference wavelength of 620 nm.

Each sample was tested in triplicate within the same test run and mean optical density (OD) values for each sample were converted to a percentage of the positive control (PP) values by using the following equation: (mean of net OD of test sample/mean of net OD of positive control) × 100. The PP value frequency distribution resulting from analyses of serum samples from control animals was determined to be normal by using the χ^2 goodness-of-fit test. Cutoff values were determined by adding 3 × SD to the mean of the PP values obtained from analyses of negative control serum samples. The resulted cutoff values were 15.6, 15.3, 14.4, 13.7, and 14.2 for sheep, cattle, dog, pig, and chicken serum samples, respectively. A sample was considered positive if the PP value was above the cutoff threshold. For the detection of antibodies in a dog that was found positive with viral RNA in the blood, an indirect IgG ELISA was used. Briefly, the plate was coated with purified N protein as described, 2-fold dilutions of the serum samples were added, followed by HRP-conjugated anti-dog IgG (Sigma, Saint Louis, MO, USA). Cutoff values for the assay were determined by adding 3 × SD to the mean of OD values resulting from analyses of negative control serum samples from non-infected dogs. Endpoint titers were expressed as the reciprocal of the highest dilution of the serum samples.

Neutralization Assay

A microneutralization assay was performed to detect neutralizing antibodies against SFTSV as described (*1*). Serial dilutions of sheep serum samples collected at various time points were mixed with an equal volume of 100 50% tissue culture infective doses of SFTSV (strain HB29), and the mixture was incubated at 37°C for 1.5 h. The mixture was then added to cultured Vero cells in a 96-well plate in quadruplicate. The plates were incubated at 37°C with 5% CO_2 for 7 days. Viral infection was assessed by an immunofluorescence assay with mouse polyclonal antibodies against SFTSV. End-point titers were expressed as the reciprocal of the highest dilution of the serum that prevented infection.

TaqMan Quantitative Real-Time Reverse Transcription–PCR

A TaqMan quantitative real-time reverse transcription PCR (qRT-PCR) was performed on all animal serum samples by using a certified real-time RT-PCR kit for clinical diagnosis (SFDA registration no. 340166, China). This kit targets the small segment of SFTSV, with 98.6% sensitivity and 99.1% specificity as described (*7*). Viral RNA copy numbers were determined from amplification of a standard curve of positive control RNA, and the cutoff cycle threshold (C^t) value for a positive reaction was set at 35 cycles. The detection limit for this kit is 50 RNA copies (5 mL/test) or 10 copies/mL. A result was considered positive if a sample had a C^t value below the cutoff.

Table. Summary of SFTS virus viral RNA and specific antibodies detected in domesticated animals, China, 2011*

Animal hosts	Laizhou Province			Penglai Province			Total		
	No. (%) RNA positive†	No. (%) antibody positive	No. serum samples tested	No. (%) RNA positive†	No. (%) antibody positive)	No. serum samples tested	No. (%) RNA positive†	No. (%) antibody positive	No. serum samples tested
Sheep	7 (3.4)	142 (69.3)	205	11 (4.1)	186 (69.7)	267	18 (3.8)	315 (69.5)	472
Cattle	22 (5.1)	233 (53.7)	433	13 (3.2)	276 (67.5)	409	35 (4.2)	442 (60.4)	842
Dogs	12 (6.6)	70 (38.5)	182	7 (4)	66 (37.3)	177	19 (5.3)	136 (37.9)	359
Pigs	13 (3.1)	3 (0.7)	426	9 (2.2)	23 (5.6)	413	22 (2.6)	26 (3.1)	839
Chickens	7 (2.7)	149 (57.1)	261	2 (0.8)	101 (38)	266	9 (1.7)	250 (47.4)	527

*SFTS, severe fever with thrombocytopenia syndrome virus.
†Serum from sampled animals of each species was collected from April to November 2011.

Virus Isolation and Sequence Analysis

Serum samples collected from animals or SFTS patients in acute phase, newly occurred in the study region in 2011, were inoculated onto monolayers of Vero cells for virus isolation as described (*1*). Cells were cultured at 37°C in an incubator supplied with 5% CO_2 with media changes done twice per week. The isolated virus was sequenced and subjected to phylogenetic analysis by using the neighbor-joining method with MEGA version 5 (*8*), and compared with previous published viral sequences of SFTSV strains isolated from 11 SFTS patients and *H. longicornis* ticks in 2010 (*1*,*3*).

Statistical Analyses

The rates of SFTSV RNA and antibody detection in Penglai and Laizhou were calculated monthly by animal species, and the statistical significance was analyzed by using either the Pearson χ^2 or continuity correction χ^2 test. Analyses were performed by using the SPSS software version 16.0 (IBM, Armonk, NY, USA). Viral RNA copies among animal species were analyzed by using the Kruskal–Wallis test, performed by using GraphPad Prism software (ver. 5.0; GraphPad Software, Inc., San Diego, CA, USA). Values of $p<0.05$ were considered statistically significant.

Ethical Considerations

According to the medical research regulation of the ministry of health, all studies involved in human samples were reviewed and approved by the ethics committee of China CDC, which uses international guidelines to ensure confidentiality, anonymity, and informed consent. Written informed consent was provided by the patients. The protocols for animal sampling have been approved by the animal care committee of China CDC.

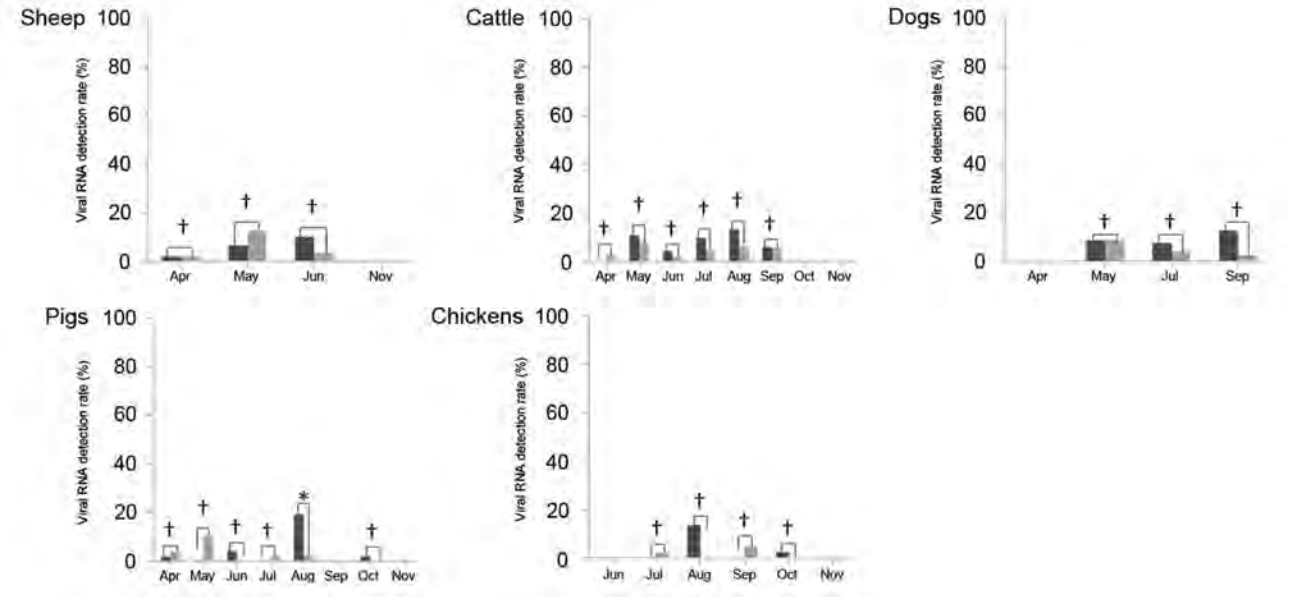

Figure 1. Serum severe fever with thrombocytopenia syndrome virus RNA detection rate in domestic animals from Laizhou and Penglai counties, China, April–November 2011. Viral RNA copies were detected by real-time reverse transcription PCR in serum samples from sheep, cattle, dogs, pigs, and chickens collected from Laizhou and Penglai counties in different months. The viral RNA detection rates are shown. Black bars indicate samples from Laizhou; gray bars indicate samples from Penglai. The viral RNA detection rate in 2 counties was compared (*$p<0.05$, †$p>0.05$).

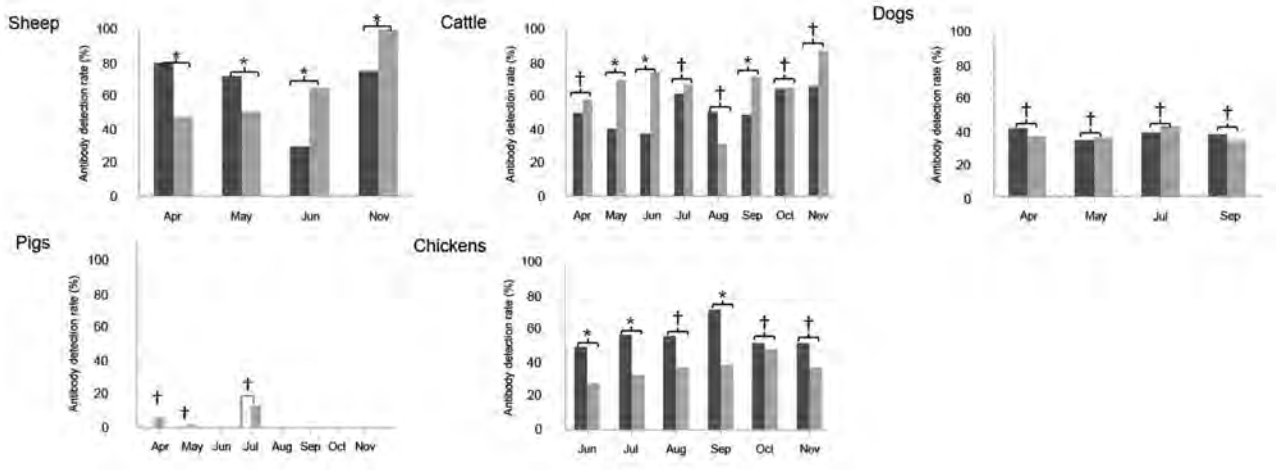

Figure 2. Serum antibody detection rate in domestic animals from Laizhou and Penglai counties, China, April–November 2011. Severe fever with thrombocytopenia syndrome virus N protein-specific antibodies were detected by double-antigen sandwich ELISA in serum samples of sheep, cattle, dogs, pigs, and chickens collected from Laizhou and Penglai counties in different months, and the antibody detection rates are presented. Black bars indicate samples from Laizhou; gray bars indicate samples from Penglai. The antibody detection rates in the 2 counties were compared (*p<0.05, †p>0.05).

Results

SFTSV Infection in Domesticated Animals by Month

We sampled 472 sheep, 842 cattle, 359 dogs, 839 pigs, and 527 chickens in Laizhou and Penglai counties to assess the prevalence of SFTSV RNA and antibodies. Our results showed that 3.8% (18/472) of sheep, 4.2% (35/842) of cattle, 5.3% (19/359) of dogs, 2.6% (22/839) of pigs, and 1.7% (9/527) of chickens were viral RNA-positive (Table). The monthly positive rates of viral RNA in sampled animals varied from April to November, ranging from 2% (n = 51) to 12.7% (n = 55) in sheep, 2.1% (n = 51) to 13.2% (n = 53) in cattle, 0 (n = 53) to 12.5% (n = 40) in dogs, 0 (n = 51) to 19.1% (n = 47) in pigs, and 0 (n = 40) to 13.5% (n = 37) in chickens, in which n is referred as to denominator of all animals sampled in that month (Figure 1). Although the overall positive rates for SFTSV varied between the 2 counties, the variance was not statistically significant (p>0.05). The median RNA concentration was 2.0×10^4 copies/mL (95% CI: $1.6–2.6 \times 10^4$) in sheep; 2.0×10^4 copies/mL (95% CI: $1.5–2.9 \times 10^4$) in cattle; 2.1×10^4 copies/mL (95% CI: $0.96–4.7 \times 10^4$) in dogs; 1.7×10^4 copies/mL (95% CI: $1.4–2.1 \times 10^4$) in pigs; and 1.7×10^4 copies/mL (95% CI: $1.0–2.7 \times 10^4$) in chickens (online Technical Appendix Figure 2), and the differences among the studied animal species were not statistically significant (p = 0.87) (online Technical Appendix Figure 2).

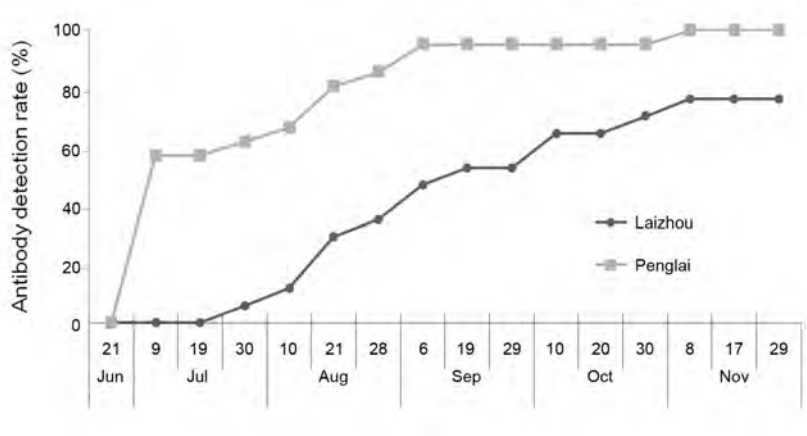

Figure 3. Detection of serum severe fever with thrombocytopenia syndrome virus (SFTSV) RNA and antibodies in a cohort of 38 sheep, China. Serum samples in a cohort of 38 sheep were collected from Laizhou and Penglai on the dates indicated on the x-axis from June 21 through November 29, 2011. SFTSV N protein–specific antibodies were measured by using a double-antigen sandwich ELISA, and the cumulative positive percentage in Laizhou and Penglai counties is presented along the timeline.

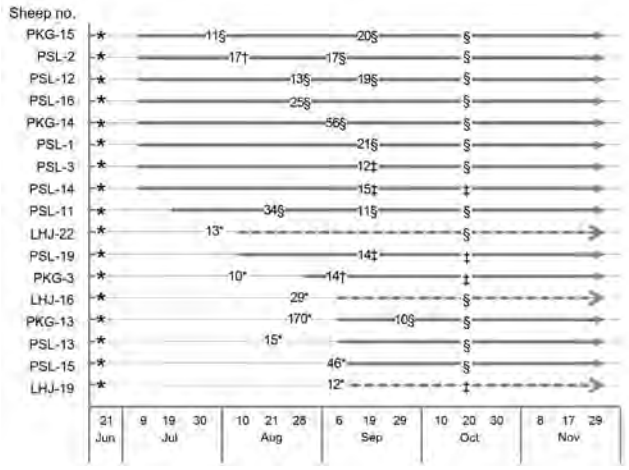

Figure 4. Time course of serum viral RNA and neutralizing antibodies in 17 sheep positive for severe fever with thrombocytopenia syndrome virus RNA, China. Detection of virus specific antibodies in Laizhou and Penglai is indicated by the dashed line and the bold line, respectively. The numbers on the lines indicate viral RNA copies (×10^3/mL) in serum samples detected on the indicated dates. Neutralizing antibody levels in the initial samples collected on June 21, 2011, in the samples positive for viral RNA, and in the samples collected at the late stage on October 20, were measured by using a microneutralization assay. Neutralizing antibody titers are shown with different symbols: *neutralizing antibodies not detected; †neutralizing antibody titers = 4; ‡neutralizing antibody titers = 16; §neutralizing antibody titers >64.

Viral antibody prevalence was significantly higher ($p<0.005$) in sheep (69.5%, 328/472), cattle (60.4%, 509/ 842), dogs (37.9%, 136/359), and chickens (47.4%, 250/527) than in pigs (3.1%, 26/839) (Table; online Technical Appendix Figure 3). As shown in Figure 2, monthly positive rates of antibodies in sheep varied from 30% (n = 30) to 80.4% (n = 51) in Laizhou County, and 48% (n = 50) to 100% (n = 82) in Penglai County during the sampling period; monthly positive rates in cattle varied from 37.5.3% (n = 48) to 66.3% (n = 80) in Laizhou, and from 31.3% (n = 48) to 78.7% (n = 87) in Penglai. In dogs, monthly positive rates ranged from 34.2% (n = 41) to 42.6% (n = 47) in the 2 regions, and the variation between sampling months was not statistically significant. Seroprevalence in pigs was low, and SFTSV antibodies were not detected in pig serum samples collected in August and September (n = 181). Monthly seroprevalence ranged from 28% (n = 25) to 72.7% (n = 44) in chickens in the regions.

SFTSV Infection in a Cohort of 38 Sheep

In addition to the 472 sheep described above, a cohort of 38 sheep was monitored for SFTSV seroconversion during a 6-month period. As shown in Figure 3, seroconversion occurred in the cohort during the surveillance period, with an increasing seroprevalence that peaked at 76.5% (n = 17) in Laizhou and 100% (n = 21) in Penglai in November. In this cohort, 17 sheep were viral RNA positive either once or twice (6/17) over the sampling period. Viral RNA was mainly detected in August and September, and serum virus RNA quantities ranged from 10^4 copies/mL to 1.7 × 10^5 copies/mL (Sheep PKG-13). In 5 sheep, viral RNA appeared before seroconversion; but in 2 sheep (PSL-15, LHJ-19), viral RNA and SFTSV antibodies were detected on the same sampling day. In 10 of 17 sheep, viral RNA was detected once or twice after seroconversion (Figure 4).

Neutralizing antibody levels were also measured in 17 sheep serum samples on day 1 (June 21) and on the day when viral RNA was first detected, and again at a later stage (October 20). All tested samples were negative for neutralizing antibodies on day 1 but were positive at later times (Figure 4). No visible clinical signs of infection were detected in the cohort of sheep over the testing period regardless of antigen or antibody status.

Course of SFTS Virus Infection in a Dog

During the testing, we found 1 dog with a high viral RNA load (1.7 × 10^7 copies/mL) and no antibodies. The dog was isolated, examined, and sampled daily from day 8 through day 22 and a sampled once more on day 90 (Figure 5). This dog had detectable viral RNA on days 8 and 10; levels declined thereafter, and levels were undetectable levels on day 12. Concurrently, SFTSV IgG titers reached 1:2,560 on day 10 and remained at that level through day 90. No signs of illness were observed during the entire period of isolation.

Virus Isolation and Sequence Analysis

Virus isolation was attempted on all viral RNA–positive serum samples (n = 103), but isolates were successfully obtained from only 1 sheep (Laizhou), 1 cattle (Laizhou), and 1 dog (Penglai), named SDLZSheep01/2011, SDLZCattle05/2011, and SDPLDog01/2011, respectively. In addition, 10 viral isolates were obtained from SFTS patient serum samples collected in 2011, 9 were from Laizhou (SDLZP01–09/2011) and 1 was from Penglai (SDPL01/2011). Phylogenetic analysis of the S segment of these SFTSV isolates indicated that the viral isolates from sheep, cattle, and dog are genetically close to the 10 SFTS patient-derived isolates in 2011 from the study areas and also to the previous published sequences of viral isolates from 11 SFTS patients reported in 2010 in 6 provinces and *H. longicornis* obtained in 2010 in Laizhou (Figure 6). Pairwise distances analysis showed that all sequences of the isolates from domesticated animals, human patients, and *H. longicornis* shared more than 95% identity, which demonstrated a close evolutionary relationship among those SFTSV isolates from domesticated animals, ticks, and SFTS patients.

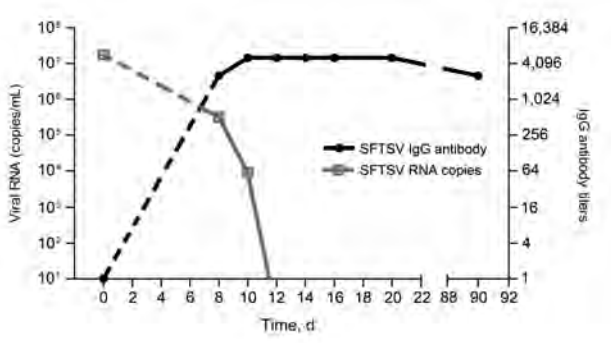

Figure 5. Time course of serum severe fever with thrombocytopenia syndrome virus (SFTSV) RNA and antibody in a naturally infected dog, China, 2011. SFTSV RNA copies and virus-specific antibodies were detected in serum samples from a dog on day 0, once every sample period of 2 days from day 8 to day 22, and on day 90. The gray open squares indicate viral copies; black circles indicate virus-specific IgG. The dashed lines indicate predicted time course of viral RNA and antibodies due to lack of data during day 1–day 7.

Discussion

In our study of >3,000 serum samples collected from 5 species of domesticated animals in 2 SFTS-endemic counties of Shandong Province, we provided evidence of natural infection and circulation of SFTSV in these animals. We found especially high (>60%) seropositivity of SFTSV-specific antibodies in sampled sheep and cattle, while almost one-half of chickens and one third of dogs tested were seropositive. We detected SFTSV RNA in all 5 species of domesticated animals at a level ranging from 1.7% to 5.3%. A cohort study with 38 sheep and a follow-up study of an infected dog provided insights into the kinetics of natural SFTSV infections in these species. Moreover, the isolation of SFTSV from a sheep, cattle, and a dog provided evidence of SFTSV viremia in domesticated animals.

A previous study in Yiyuan County, Shandong Province, showed a high seroprevalence in goats (83%) (*4*), and another serosurvey of domesticated animals conducted in Jiangsu Province found SFTSV antibody–positive rates of 57% in goats, 32% in cattle, 6% in dogs, 5% in pigs, and 1% in chickens but no antibodies in geese and mice (*5*). Our investigation, conducted in 2 other SFTS-endemic regions in China (online Technical Appendix Figure 1), demonstrates a high seroprevalence of SFTSV in sheep (69.5%), cattle (60.4%), dogs (37.9%), and chickens (47.4%), but low rates in pigs (3.1%). Results of seroprevalence of SFTSV infection were consistent and especially high in goats and sheep but low in pigs in these studies. However, our data showed a higher seropositivity of SFTSV-specific antibodies in cattle, dogs, and chickens than the previous report in Jiangsu Province.

In addition, we also found that the SFTSV RNA was detected in all sampled animal species, but the prevalence was low, ranging from 1.7% to 5.3%, which indicates the potential viremia in these animals. The differences in the rates of SFTSV infections among various animal species and regions were statistically significant, perhaps because of varying degrees of exposure to virus-infected ticks or differences in host susceptibility to SFTSV infection. The sheep and cattle in our study regions were grazed on pastures or hills during the day and kept in household backyards at night; the dogs and chickens roamed freely in fields. Pigs were the only animals that did not roam freely. We found that the animals in this study, particularly the sheep and cattle, were heavily infested with ticks. *H. longicornis*, which has been implicated as a vector of SFTSV, is the dominant tick species in these areas. SFTSV RNA had been detected by using a method of real-time RT-PCR from *H. longicornis* ticks collected from grass, cattle, and sheep with a detection rate of ≈2% in disease-endemic areas in previous studies (*3*,*9*), and a viral isolate was obtained from *H. longicornis* ticks on sheep from the same study area of Laizhou in 2010 (*3*). Phylogenetic analysis of the virus isolates obtained from the sheep, cattle, and dog in this study and from *H. longicornis* ticks in our previous study (*3*) showed >95% homology with SFTSV isolates obtained from patients from the same region, which suggests a potential link of SFTSV infections among humans, domesticated animals, and ticks.

Infective virus, a competent insect vector, and susceptible vertebrate hosts must coexist to establish and maintain arbovirus transmission cycles (*10*). Threshold viremia levels of $10^{2.0–4.7}$ 50% lethal dose/mL have been reported as sufficient for the infection of ticks with Colorado tick fever virus, Russian spring-summer encephalitis virus, and louping ill virus (*11–14*). In our study, we detected viral RNA in all domesticated animal species examined, although the viral RNA copy numbers were low ($<10^5$ copies/mL). The individual dog in which the clinical course of infection was followed showed a course of acute infection typical of a bunyavirus infection, including the stages of viremia and antibody response. Successful isolation of SFTSV from a sheep, cattle, and a dog confirms the occurrence of SFTSV viremia in these domesticated animals. However, only 3 isolates were obtained from 103 SFTSV RNA–positive serum samples, which may indicate that most infected domesticated animals might have either a low level of viremia, a short period of viremia, or that few infectious virions might be present under the condition of high level of serum antibodies.

During the cohort study of 38 sheep, seroconversion was observed, indicating ongoing circulation of SFTSV in the endemic areas. In 17 of 38 sheep, neutralizing antibodies were observed in the viral RNA in the blood. Although more evidence is needed to illustrate the underlying mechanisms, this case is not the only case of

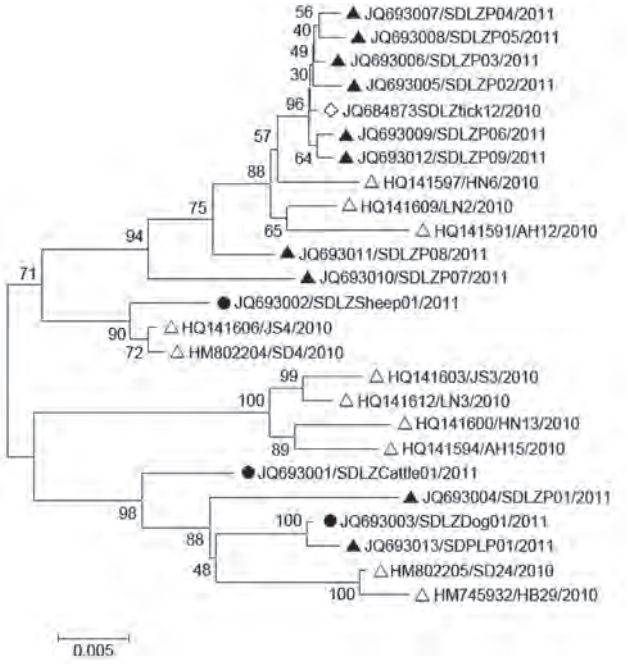

Figure 6. Phylogenetic analysis of severe fever with thrombocytopenia syndrome virus (SFTSV) isolates from domesticated animals. The evolutionary relationship of small segments of SFTSV isolated from domesticated animals, SFTS patients and ticks was calculated by using the neighbor-joining method with MEGA 5 (*8*). Sequences are labeled with the order of GenBank accession number/name of viral strain/year of isolation. Black circles indicate the original sequences of SFTSV strains obtained from domesticated animals in this study; black triangles indicated the original sequences of SFTSV strains obtained from SFTS patients in 2011 in this study; blank triangles indicate the previously published sequences of SFTSV strains obtained from 11 SFTS patients in 2010 (*1*); and open diamonds indicate the previously published sequences of SFTSV strains obtained from H. longicornis ticks in 2010 (*3*). Scale bar indicates nucleotide substitutions per site.

Bunyaviradae virus infection. A persistent infection of hantavirus can be established in the rodent reservoir, lasting several months or years with high titers of neutralizing antibodies in the serum (*15*,*16*). Persistent infection of SFTSV in animals has not been shown to date, and a more sensitive nucleotide acid detection method may help to define whether SFTSV infection is prolonged in domestic animals. However, the evidence suggests that SFTSV is circulating among animals and between animals and humans in the endemic areas.

The results of this study are subject to several limitations. First, only 3 viral isolates (1 from each animal species) were obtained and sequenced, which limits the ability to generalize the representativeness of SFTS viral diversity in these areas. Second, SFTSV infections in ticks on animals were not systematically studied to evaluate their relation to SFTSV-infected status or with seroconversion of studied animals, which makes it difficult to associate the infection of SFTSV in ticks directly with that in animals and human patients. Third, we cannot determine whether the virus is pathogenic to the domesticated animals in this study because no adequate examinations have been taken place.

In conclusion, we have provided evidence to show that SFTSV is circulating among several species of domesticated animals and between animals and humans in disease-endemic areas of China. The domesticated animals with high infection rates of SFTSV may act as amplifying hosts for the virus during the epidemic season. More studies are needed to elucidate the SFTSV transmission model in nature, which includes determining the duration of viremia levels and the possibility of persistent infection in each animal species, potential reservoirs, and links of SFTSV infections among humans and domesticated animals. These findings will help formulate effective measures for containing the infection of this emerging pathogen.

Acknowledgments

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Mr Niu is a PhD candidate under the supervision of Dexin Li, in the National Institute for Viral Diseases Control and Prevention, China CDC. His research interest is focused on epidemiologic investigation of SFTSV infections in animal hosts.

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Campylobacter coli Outbreak in Men Who Have Sex with Men, Quebec, Canada, 2010–2011

Christiane Gaudreau, Melissa Helferty, Jean-Loup Sylvestre, Robert Allard, Pierre A. Pilon, Michel Poisson, and Sadjia Bekal

During September 2010–November 2011, a cluster of erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant *Campylobacter coli* pulsovar 1 infections was documented, involving 10 case-patients, in Montreal, Quebec, Canada. The findings suggested sexual transmission of an enteric infection among men who have sex with men.

Campylobacter coli is the second most common species that causes human *Campylobacter* infections (*1–3*). Few studies have characterized the differences between the epidemiology and the disease of *C. coli* infections in comparison to *C. jejuni* subsp. *jejuni* infections (*1–3*). However, many studies have reported a higher macrolide resistance in *C. coli* than in *C. jejuni* (*1–3*). Few *C. coli* outbreaks have been reported to date (*4*,*5*).

The Study

A retrospective analysis, including the period from January 1, 2010 through December 31, 2011, identified 43 laboratory-confirmed cases of *C. coli* infections reported to the Montreal Public Health Department; among them, 40 cases with antimicrobial drug susceptibility results were further analyzed. Telephone interviews with the case-patients were conducted by using a standardized questionnaire pertaining to symptomatology of the illness, treatment, exposures, sexual orientation (including practices), and HIV status. The questionnaire was mailed to persons who could not be contacted by phone. Hospital charts for 9 or the 10 outbreak case-patients were reviewed retrospectively.

Statistical analyses, using Fisher exact test to calculate the possibilities, were conducted to test for differences in characteristics between case-patients infected with the outbreak etiologic agent, *C. coli* pulsovar 1, and those infected with nonoutbreak *C. coli*. In estimating the odds ratio from a 2 × 2 table that included a zero cell, 0.5 was added to the count in each cell. CIs were calculated by using Miettinen's test-based method. Statistical analyses were conducted using SPSS software (http://www-01.ibm.com/software/analytics/spss/products/statistics/).

Phenotypic identification of *Campylobacter* isolates at the genus and species levels was confirmed by *cpn60* gene sequencing at Laboratoire de Santé Publique du Québec. *C. coli* strains were identified by direct sequencing of PCR-amplified partial *cpn*60 sequences as described by Hill et al. (*6*). DNA sequences were determined with an ABI 3100 sequencer using a BigDye sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences were subjected to a BLAST analysis and aligned with the ClustalW program. Phylogenetic analysis was performed using the Lasergene software V6.1 (DNAstar, Madison, WI, USA).

Genetic relatedness was investigated by using pulsed-field gel electrophoresis with *Sma*I according to PulseNet Canada procedures. *Salmonella enterica* serotype Braenderup strain H9812 was used as the marker size in each gel (*7*). For analysis, band position tolerance and optimization values of 1% were used. Similarity coefficient was obtained with the unweighted pair-group method with arithmetic averages. For strains exhibiting similar patterns with *Sma*I, a second enzyme (*Kpn*I) was used to confirm their pulsed-field gel electrophoresis pattern similarity. The PulseNet Canada *Sma*I and *Kpn*I pattern designations for the *C. coli* pulsovar 1 isolate are CASAI.0160 and CAKNI.0078, respectively.

Antimicrobial drug susceptibility testing was determined by using the disk diffusion method for erythromycin, tetracycline, and ciprofloxacin (*8*) and the Etest (AB Biodisk, Solna, Sweden) method for all 12 agents tested (*3*). β-lactamase susceptibility was determined as reported (*9*).

From September 2010 through November 2011, in Montreal, 10 men, 26–57 years of age, were found to be infected with an erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant *C. coli* pulsovar 1; these men were defined as the outbreak-associated case-patients (Figure 1). An additional 5 women and 4 men were infected with an

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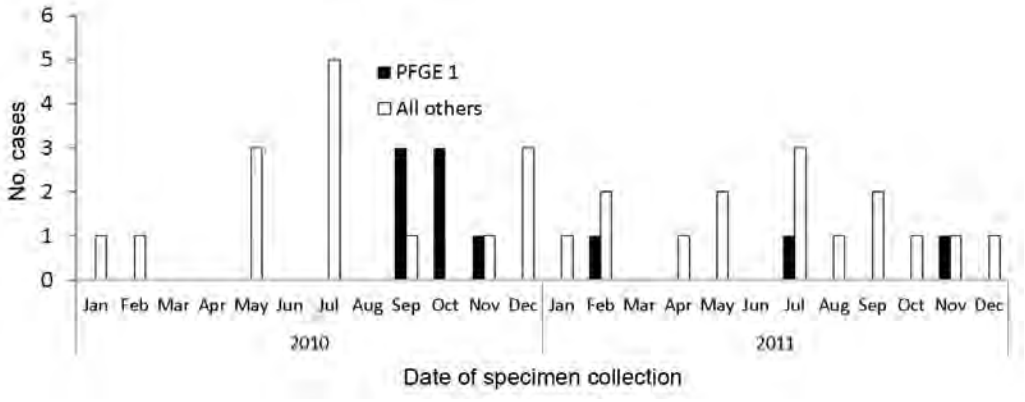

Figure 1. Number of cases of *Campylobacter coli* infection reported to Montreal Public Health, Quebec, Canada, 2010–2011. PFGE, pulsed-field gel electrophoresis.

erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant *C. coli* strain; however, 9 different pulsovars were involved (Figure 2). Microbiology laboratories at Centre Hospitalier de l'Université de Montréal documented 9 of the 10 outbreak cases, but did not isolate erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant *C. coli* from December 2011 through November 2012.

Compared with the 30 nonoutbreak case-patients for whom suceptibility results were available, the 10 outbreak case-patients were more likely to be male (p = 0.010), to be 20–59 years of age (p = 0.010), to be men who have sex with men (MSM) (p = 0.0001), to be HIV positive (p = 0.001), and to have had sexual relations within 2 weeks of the beginning of gastrointestinal symptoms (p = 0.017) (Table 1). Of the 8 HIV-positive patients, the CD4 cell count was 210 $\times 10^6$/L for 1 man and 440–1,150 $\times$ 10^6/L for the 6 other patients, and the HIV viral load was 68 copies/mL for 1 patient and <40 copies/mL for the 5 other patients for whom these data were known.

Seven men (70%), all MSM, lived in surrounding neighborhoods of Montreal's so-called Gay Village. The antimicrobial drug treatment regimen was known for 1 outbreak case-patient who received oral azithromycin. In the previous 15 years, 1 HIV-positive man (who also had sex with men) and had *C. coli* pulsovar 1 and *S. flexneri* in fecal specimens, exhibited 7 other sexually transmitted diseases. Among the 10 outbreak case-patients, 2 HIV-positive MSM were simultaneously infected with an *S. flexneri* isolate, and 1 of these 2 patients experienced *C. coli* septicemia.

The following data were reported for 10 outbreak *C. coli* and 30 nonoutbreak *C. coli* case-patients, respectively: diarrhea, 100% (6/6) and 88% (23/26); abdominal cramps, 60% (3/5) and 83% (19/23); blood in stool specimen, 20% (1/5) and 30% (6/20); fever, 60% (3/5) and 61% (14/23); and hospitalization, 17% (1/6) and 19% (5/26) (p>0.05 for all data). Exposures to potential sources of infection did not differ between outbreak and nonoutbreak case-patients. All patients reported having consumed meat, dairy products, tap water or commercially bottled only, and no nonchlorinated water. Exposures to animals, farms, and other persons with known cases of diarrhea were rarely reported. Travel history outside of the island of Montreal in the 2 weeks before symptom onset was documented in none (0/6) of *C. coli* pulsovar 1 case-patients and in 48% (12/25) of other *C. coli* case-patients (p = 0.059); all 12 had traveled outside Canada.

The 10 outbreak isolates were susceptible to erythromycin, azithromycin, ampicillin, gentamicin, imipenem, clindamycin, chloramphenicol, and tigecycline and were β-lactamase negative. All 10 were resistant to ciprofloxacin, nalidixic acid, tetracycline, and cefotaxime (Table 2).

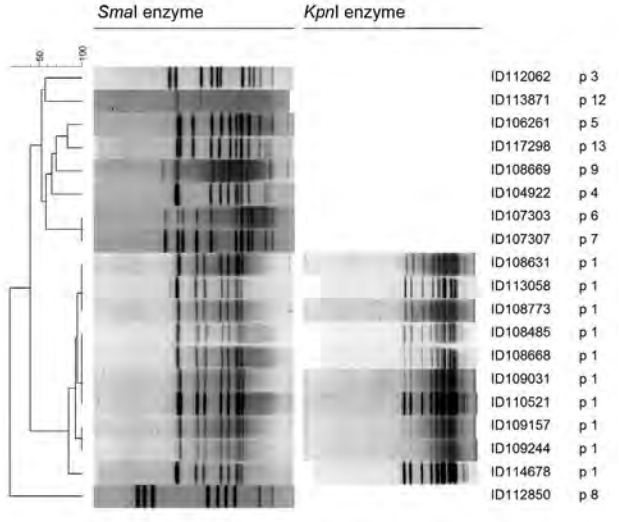

Figure 2. Pulsed-field gel electrophoresis (PFGE) patterns of erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant, *Campylobacter coli* for *SmaI* (19 isolates) and *KpnI* (10 isolates) enzymes, Montreal, Quebec, Canada, 2010–2011. Scale bar indicates percent similarity. p, pulsovar.

Table 1. Statistically significant differences between case-patients with outbreak-related *Campylobacter coli* pulsovar 1 (n = 10) and nonoutbreak *C. coli* (n = 30) infections, Montreal, Quebec, Canada, 2010–2011*

Characteristic	No. outbreak case-patients	No. nonoutbreak case-patients	OR (95% CI)	p value
Male sex, n = 40	10/10	17/30	16.20 (1.88–139.00)	0.010
Age 20– 59 y, n = 40	10/10	17/30	16.20 (1.88–139.00)	0.010
Had sexual relations within the incubation period, n = 23	6/6	7/17	18.20 (1.87–177.00)	0.017
MSM, n = 17	9/10	0/7	95.00 (8.29–1,089.00)	0.0001
HIV positive, n = 15	8/9	0/6	73.67 (6.09–891.00)	0.001

*OR, odds ratio; MSM, men who have sex with men.

Conclusions

Epidemiologic and molecular data confirmed a cluster of erythromycin-susceptible, tetracycline- and ciprofloxacin-resistant, *C. coli* pulsovar 1 infections in MSM in Montreal, Quebec, Canada, during September 2010–November 2011. The epidemiologic data reported in Table 1, the 14-month outbreak duration, the simultaneous *S. flexneri* infection in 2 HIV-positive MSM, and the absence of any reported common food exposure suggest a sexually transmitted enteric infection. A cluster of erythromycin- and ciprofloxacin-resistant, tetracycline-susceptible *C. jejuni* subsp. *jejuni* infections from 1999 through 2001 (*13*) and 7 clusters of *Shigella* spp. infections from 1999 through 2011 (*14*,*15;* unpub. data), which were sexually transmitted, have been documented in MSM in Montreal and surrounding neighborhoods. Among MSM, *Shigella* spp. infection is, in most cases, sexually transmitted (*15*).

C. coli infection clusters are infrequently reported (*4*,*5*). *Campylobacter* should be identified to species level by phenotypic and, if needed, by molecular characterization. Association of cluster cases with the correct *Campylobacter* species is the first step of suspecting an outbreak and can lead to improved outbreak detection. Antimicrobial drug susceptibility testing, at least to erythromycin and ciprofloxacin, is recommended for every isolate (*10*). The erythromycin, ciprofloxacin, and tetracycline susceptibilities were epidemiologic markers in the *Campylobacter* spp. clusters documented in Montreal (present study; *13*). Nine different pulsovars were documented in 9 nonoutbreak case-patients, indicating a high heterogeneity of *C. coli*.

If necessary, the first-choice antimicrobial drug treatment for patients infected with *C. coli* pulsovar 1 would be a macrolide as it is for *C. jejuni* and *C. coli* enteric infections because of increasing fluoroquinolone resistance in these bacteria (*1*–*3*). HIV-positive or AIDS patients may have a higher incidence of *Campylobacter* infections with more septicemia and more complicated outcome than healthy patients have (*1*,*2*). MSM should be counseled on methods to avoid or reduce the risk of sexual transmission of enteric infections such as those caused by *Campylobacter* or *Shigella* (*13*).

Table 2. Antimicrobial drug susceptibility results for *Campylobacter coli* pulsovar 1 isolates from 10 patients, Montreal, Quebec, Canada, 2010–2011*

Antimicrobial agent†	MIC (mg/L)	Interpretation
Erythromycin	2–4	S
Azithromycin	0.25–0.5	S
Tetracycline	128–256	R
Ciprofloxacin	>32	R
Nalidixic acid	>256	R
Ampicillin	2–4	S
Gentamicin	0.5–1	S
Cefotaxime	>32	R
Imipenem	0.06–0.12	S
Clindamycin	0.25–0.5	S
Chloramphenicol	2–4	S
Tigecycline	≤0.015	NA
β-lactamase‡	Negative	–

*S, susceptible; R, resistant; NA, not available; –, not applicable.
†The susceptibility and resistance breakpoints were Clinical and Laboratory Standards Institute (CLSI) *Campylobacter* breakpoints for erythromycin, tetracycline and ciprofloxacin (*10*), National Antimicrobial Resistance Monitoring System *Campylobacter* breakpoints for azithromycin and clindamycin (*11*), no breakpoints available for tigecycline and CLSI *Enterobacteriaceae* breakpoints for the 6 other antimicrobial agents (*12*).
‡β-lactamase susceptibility was determined as described (*9*).

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Delayed Diagnosis of Chronic Q Fever and Cardiac Valve Surgery

Linda M. Kampschreur, Elske Hoornenborg, Nicole H. M. Renders, Jan Jelrik Oosterheert, Joost F. Haverman, Peter Elsman, and Peter C. Wever

Untreated chronic Q fever causes a high number of complications and deaths. We present cases of chronic Q fever that were not diagnosed until after the patients underwent cardiac valve surgery. In epidemic areas, Q fever screening of valve surgery patients secures early initiation of treatment and can prevent illness and death.

Q fever, a zoonosis caused by the intracellular gram-negative bacterium C*oxiella burnetii*, occurs in outbreaks and is prevalent worldwide. Q fever has acute and chronic stages (*1*). Acute Q fever is a self-limiting febrile disease occurring in 40%–50% of *C. burnetii*–infected persons (*1*). Chronic Q fever can develop years after primary infection and occurs in 1%–5% of *C. burnetii*–infected persons (*1*,*2*). The most critical manifestations of chronic Q fever are endocarditis and infections of vascular prosthesis and aortic aneurysms (*3*). Persons with pre-existing valvular cardiac disease have a reported 40% risk of Q fever endocarditis when infected with *C. burnetii* (*2*,*4*).

During 2007–2010, an outbreak of >4,000 cases of acute Q fever occurred in the Netherlands (*5*). To increase understanding of the role of Q fever in valvular cardiac disease, we present 3 cases of chronic Q fever and valvular cardiac disease requiring surgery in patients from the Netherlands. The diagnosis of chronic Q fever was not made until after the patients had elective cardiac valve surgery for progressive valvular dysfunction.

Case 1

In 2004, aortic valve stenosis of a tricuspid valve was diagnosed in a 73-year-old man. Additional medical history included atrial fibrillation and transient ischemic attacks. Because of progressive stenosis, the patient underwent aortic valve replacement with a bioprosthesis in May 2011. The removed valve had no macroscopic signs of endocarditis, so neither microbiological nor pathological examination was requested. Four months later, paravalvular insufficiency of the bioprosthesis developed in the patient, requiring a second valve replacement. Transesophageal echocardiography revealed no vegetations. Macroscopic signs of endocarditis were not observed on the removed valve; further examination was not requested. However, serologic testing for *C. burnetii* revealed chronic infection (Table).

The patient had not been aware of previous acute Q fever infection and had not experienced fever, night sweats, weight loss, or malaise. Further examination by fluorodeoxyglucose positron emission tomography (FDG PET) combined with low-dose computed tomography (CT) demonstrated no other chronic Q fever focus or vascular abnormalities. The patient started antimicrobial drug therapy (doxycycline and hydroxychloroquine) and was doing well 3 months later. Retrospective microbiological examination of a serum sample obtained at the time of the first valve replacement demonstrated a profile consistent with chronic Q fever (Table).

Case 2

A 78-year-old man had a medical history of aortic valve stenosis of a tricuspid valve, abdominal aortic aneurysm, and endovascular aneurysm repair in 2005. In July 2011, he was screened for chronic Q fever in a program for patients at high risk for development of chronic Q fever (e.g., persons with a vascular prosthesis or aneurysm) (*6*); the screening revealed that he did have chronic Q fever infection (Table). The patient had not been aware of an acute Q fever episode and did not report night sweats, weight loss, malaise, or fever. Because the he had progressive aortic valve stenosis, the patient was on a waiting list for elective valve replacement at an academic cardiovascular center. This center, located outside the Q fever epidemic area and unaware of the patient's Q fever status, placed a bioprosthesis in the patient in August 2011. The native valve was not further examined because there were no macroscopic signs of endocarditis.

After the surgery, the Q fever screening results were acted upon. FDG PET/CT scan results showed no signs of infection at the abdominal aortic prosthesis or elsewhere. In September 2011, the patient started antimicrobial drug therapy (doxycycline and hydroxychloroquine) and was doing well at a 6-month follow-up visit.

Case 3

A 70-year-old woman had a longstanding history of rheumatoid arthritis that was treated consecutively

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with infliximab and etanercept plus corticosteroids and azathioprine. In 2009, she was hospitalized because of heart failure caused by mitral valve insufficiency, possibly resulting from chordal rupture, combined with an atrial septal defect and left ventricular systolic dysfunction. In October 2010, mitral valve repair, a coronary bypass, and atrial septal defect closure were performed. The patient was registered for vaccination against *C. burnetii*, which was offered by the government to persons with aortic (endo) vascular prostheses or cardiac valve abnormalities. In April 2011, prevaccination screening results showed she was positive for chronic Q fever (Table). The patient did not recall a previous acute Q fever episode, and she had not experienced fever, night sweats, malaise, or weight loss. FDG PET/CT scan results showed no FDG uptake in the large vessels.

Transesophageal echocardiography revealed an insufficiency of the mitral valve repair. An echocardiogram was not performed immediately after the valve repair in 2010, so it could not be determined whether this insufficiency was new. No vegetations or signs of endocarditis were seen. Antimicrobial drug treatment (doxycycline and hydroxychloroquine) was started and later switched to moxifloxacin monotherapy because of elevated liver enzyme levels and severe nausea and vomiting, possibly caused by hydroxychloroquine. After 15 months of treatment, the patient still had a high level of *C. burnetii* antibody.

Conclusions

We reviewed 3 cases of chronic Q fever and valvular cardiac disease requiring surgery. The diagnosis of chronic Q fever was not made until after the elective surgery. Early diagnosis and antimicrobial drug treatment of Q fever endocarditis might have prevented surgery. Symptoms of Q fever endocarditis can be nonspecific, and vegetations are usually absent or small (*7*). As observed in the cases presented here, C-reactive protein levels can be normal or only mildly elevated (*8*) (Table). The most frequent signs of Q fever endocarditis are a new valvular insufficiency or worsening of preexisting valvular insufficiency (*8–10*). *C. burnetii*–infected cardiac valves can appear normal on visual inspection, as demonstrated in the cases presented here, and on histologic evaluation (*11*).

Diagnosis of chronic Q fever is challenging. Chronic infection is determined on the basis of serologic testing and PCR of blood samples and, if available, tissue samples. In the absence of acute Q fever, PCR results positive for *C. burnetii* in blood or tissue prove chronic infection; however, the sensitivity of this test is only 50%–60% in patients with chronic Q fever (*12*). When cultured in cells, *C. burnetii* exhibits antigenic variation in which the virulent variant, called phase I, shifts to an avirulent variant, called phase II. During acute infection, antibodies to phase II antigens are detected first; persisting high levels of antibodies to phase II, and especially phase I antigens, are indicative of chronic Q fever (*13*). A phase I IgG titer >800 or >1,024, depending on the type of immunofluorescence assay used, has been internationally accepted for the serologic diagnosis of chronic Q fever (*14*,*15*).

Long-term antimicrobial drug treatment, preferably doxycycline plus hydroxychloroquine, is the treatment of choice for chronic Q fever. Treatment should continue for 18 months for native valves and 24 months for prosthetic valves, until a 4-fold decrease of phase I IgG titers and a complete clearance of phase II IgM are reached. If phase I IgG titers remain high or phase II IgM is detectable, treatment should be extended. The rates of morbidity and mortality among people with chronic Q fever are high, reaching >60% if treatment is delayed or not initiated. With adequate treatment, the mortality rate for Q fever endocarditis has declined to 5%. Chronic Q fever involving prosthetic valves is associated with a higher mortality rate, longer treatment, and elevated chance of complications (*9*). For the cases reported here, preoperative diagnosis of chronic Q fever might have prevented the second valve replacement in case-patient 1 and the delay in treatment initiation in case-patients 2 and 3. We advise preoperative

Table. Results of *Coxiella burnetii* and C-reactive protein testing for 3 patients with chronic Q fever and valvular cardiac disease requiring valve surgery, the Netherlands*

	Results for *C. burnetii* testing			
	IFA†			
Case-patient	Phase I IgG titer	Phase II IgG titer	PCR	C-reactive protein, mg/L
1				
Before valve surgery†	32,768	65,536	Negative	<6
After valve surgery	8,192	8,192	Positive	52
2				
Before valve surgery	16,384	16,384	Positive	22
After valve surgery	16,384	16,384	Positive	26
3‡				
After valve surgery	8,192	8,192	Positive	11

*IFA, immunofluorescence assay.
†Q fever serologic testing was performed retrospectively.
‡Serologic testing was not available before surgery.

serologic screening for chronic Q fever in all patients undergoing elective cardiac valve surgery in Q fever epidemic areas. If serologic test results are positive for *C. burnetii* antibodies, PCR of the excised valve should be performed.

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Treatment of Tularemia in Patient with Chronic Graft-versus-Host Disease

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We describe a case of human tularemia caused by Francisella tularensis subsp. holarctica in a stem cell transplant recipient with chronic graft-versus-host disease who was receiving levofloxacin prophylaxis. The infection was characterized by pneumonia with septic complications. The patient was successfully treated with doxycycline.

Tularemia is a zoonotic infection caused by the gram-negative bacterium *Francisella tularensis*. Humans are accidental hosts; infection occurs after contact with infected animals, contaminated water or soil, or invertebrate vectors (*1*). Strains of the 2 subspecies F. *tularensis* subsp. *tularensis* and F. *tularensis* subsp. *holarctica* account for virtually all infections in humans. Only rarely have strains of the subspecies *F. tularensis novicida* or the closely related species *F. philomiragia* or *F. hispaniensis* been cultured from clinical specimens (*2*).

F. tularensis subsp. *tularensis*, also referred to as type A, is found almost exclusively in North America and is the most virulent subspecies. *F. tularensis* subsp. *holarctica*, also referred to as type B, is found predominantly in Asia and Europe, but also in North America (*3*). Patients infected with *F. tularensis* have abrupt onset of fever, chills, headache, and malaise after an incubation period of 2–21 days. Additional signs and symptoms may develop, depending on the portal of entry. The most common signs and symptoms are lymphadenopathy, fever, pharyngitis, appearance of ulcers/eschars/papules, nausea and vomiting, and hepatosplenomegaly.

Antimicrobial drug therapy should be administered to patients with this suspected or confirmed diagnosis, even though spontaneous resolution may occur in 50%–95% of cases (depending on the clinical syndrome) (*4*). For severe tularemia, gentamicin is the drug of choice (5 mg/kg/d, divided into 2 doses and monitored by analysis of serum drug concentration). If available, streptomycin is a well-suited alternative agent. This approach is based on observational data evaluating frequency of cure and relapse with different antimicrobial drugs (*5*) and is currently recommended by the World Health Organization (*6*). Oral agents may be used for treatment of mild illness. Preferred agents are doxycycline or ciprofloxacin. Observational data for tetracycline have found an 88% cure rate and 12% relapse rate (*5*), although other studies have indicated that relapse might be more common in patients who received tetracycline than in those who received ciprofloxacin or aminoglycosides (*6*).

The Study

A 54-year-old man was admitted to the hematology department, Robert-Bosch-Hospital, Stuttgart, Germany, in early 2010, with fever (39.5°C), chills, and minor dyspnea that had lasted for 3 days. Four years earlier, he had received a stem cell transplant for acute myeloid leukemia, which was in first complete remission after myeloablative conditioning with total body irradiation, 12 Gy, and cyclophophamide, 120 mg/kg bodyweight. The post-transplant course was complicated by grade 3 graft-versus-host-disease of the skin and gut, multiple infectious episodes, chronic renal failure (creatinine level 3 mg/dL, glomerular filtration rate 25 mL/min, urea level 80 mg/dL), repeatedly occurring cytomegalovirus replications, and later on extensive chronic graft-versus-host-disease, necessitating continuous immunosuppressive therapy (tacrolimus, steroids), and anti-infective prophylaxis (dose-adjusted levofloxacin, 125 mg/d, posaconazole, 3 × 200 mg/d), respectively. After admission, chest radiograph revealed no abnormal findings. Blood cultures were drawn, and the patient was given empiric antimicrobial drug therapy with intravenous (IV) imipenem/cilastatin (500 mg/8 h) and full-dose levofloxacin (IV 2 × 250 mg/d) the same day.

Fever persisted, and a computerized tomography scan and a bronchoscopy were performed. The computerized tomography scan revealed a large infiltrate in the right upper lobe. In the initially drawn blood cultures, gram-negative rods were cultivated after 165 h. *F. tularensis* was suspected on the basis of biochemical identification, and the isolate was sent to the reference laboratory for tularemia for confirmation (Bundeswehr Institute of Microbiology, Munich, Germany). Subsequently (day 8 after admission), the antimicrobial drug therapy was extended to doxycycline (IV 2 ×100 mg/d). Aminoglycoside therapy was avoided because of the chronic renal failure. The patient was discharged afebrile after 16 days in improved condition.

Examination of smears originating from the positive blood cultures revealed bacteria that presented as

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Table. Susceptibility testing of the blood culture isolate (*Francisella tularensis* subsp. *holarctica*) break point analysis according to CLSI standard*

Antimicrobial agent	MIC, mg/L	Interpretation
Tetracycline	1.0	S
Ciprofloxacin	0.125	S
Levofloxacin	0.25	S
Chloramphenicol	2	S
Gentamicin	0.5	S
Streptomycin	2	S

*CLSI, Clinical and Laboratory Standards Institute; S, susceptible; R, resistant.

pleomorphic, faintly staining, gram-negative coccobacilli. An aerobic, slow-growing bacterium was recovered from chocolate agar after a 2-day incubation at 37°C in an atmosphere of 5% CO_2. Presumptive identification of the isolate with the gram-negative card on the VITEK 2XL instrument (bioMérieux, Nörtingen, Germany) indicated *F. tularensis.* The isolate was sent to the national reference laboratory for tularemia for confirmation and further characterization.

The presumptive phenotypic identification of the *F. tularensis* strain was confirmed by real-time PCR that targeted *Francisella*-specific 16S rDNA sequences (*Francisella* LightMix kit; TIP MOLBIOL, Berlin, Germany), a type B–specific real-time PCR that targeted the 23S rDNA gene, as well as 23S rDNA sequencing. Molecular analysis of the 23S rDNA sequence and phenotypic determination of macrolides susceptibility (Etest; bioMérieux) revealed that the isolate represented a strain of *F. tularensis holarctica* biovar I.

Antimicrobial drug susceptibility testing was performed according to the current recommendations of the Clinical and Laboratory Standards Institute (*7*) by using the commercially available, CE-certified MICRONAUT-S-microtiter broth dilution testing system (Merlin, Bornheim, Germany) and gave results characteristic for all *F. tularensis* strains (Table). Although the strain was susceptible for levofloxacin, according to Clinical and Laboratory Standards Institute standards, the MIC of 0.25 mg/L was at least twice as high when compared with 69 other *F. tularensis* subsp. *holarctica* strains (0.031, n = 11; 0.062, n = 54; 0.125, n = 4) (*7*). Multilocus variable number of tandem repeats analysis (*8*) demonstrated that the strain clustered with 10 additional German *F. tularensis* strains from which 3 were isolated from hares found in an area <20 km from the patient's home.

Conclusions

Because infection with *F. tularensis* or other *Francisella* species is relatively infrequent in nature, informative examples of infection in immunocompromised persons are rare. Elkins et al. provide a comprehensive review of *F. tularensis* infections in this patient collective, most of which occur in patients who have had a solid organ transplant or who have AIDS (*9*). Only 2 cases of tularemia caused by *F. tularensis* in stem cell or bone marrow transplant patients have been reported to date; 1 patient died because of severe neutropenia (*10*).

In the patient described here, tularemia was acquired while he was undergoing prophylaxis with levofloxacin, a potential active agent against *F. tularensis*. This failure of anti-infective prophylaxis was most probably related to the reduced levofloxacin concentration caused by renal-based dose adaption rather than because the strain had developed fluoroquinolone resistance. Although the strain isolated from the blood stream of the patient was susceptible to levofloxacin by an approved microdilution broth assay, heterogeneous resistance against fluoroquinolones could not be completely ruled out for methodologic reasons.

It has been well documented that *F. tularensis* can easily be rendered ciprofloxacin-resistant because of single nucleotide polymorphisms of the quinolone-resistant determining region of gyrase A, but such strains have so far never been isolated from patients with clinical cases (*11*). Although doxycycline is not considered the drug of choice for severe tularemia, treatment with IV doxycycline for 16 days was successful for this patient. Thus, doxycycline might be a useful alternative for treating cases in which aminoglycosides or fluoroquinolones cannot be applied or may have failed.

Careful and comprehensive survey and questioning of the patient did not definitely reveal the route of infection. The patient did not recall any insect bites or contact with animals. The only potential risk factor mentioned was mowing the lawn of his garden close to a forest about 9 days before onset of symptoms. The home region of the patient is thought to be highly endemic for tularemia. Although it remains unproven, this hypothesis is consistent with the molecular epidemiologic results, demonstrating that identical or nearly identical genotypes were found near the patient's home.

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Scrub Typhus Outbreak, Northern Thailand, 2006–2007

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During a scrub typhus outbreak investigation in Thailand, 4 isolates of *O. tsutsugamushi* were obtained and established in culture. Phylogenetic analysis based on the 56-kDa type-specific antigen gene demonstrated that the isolates fell into 4 genetic clusters, 3 of which had been previously reported and 1 that represents a new genotype.

Scrub typhus is a febrile disease endemic to the Asia–Australia–Pacific region, where ≈1 million cases occur annually (*1*). The causative agent of scrub typhus in this region is the gram-negative obligate intracellular bacterium *Orientia tsutsugamushi* (*2*). The bacterium maintains itself in trombiculid mites, and small mammals serve as reservoir hosts in the natural life cycle of the mites. Chiggers, the larval stage of mites, act as the transmission vector for *O. tsutsugamushi* (*1*). Humans and small animals become infected following the bite of chiggers harboring *O. tsutsugamushi*. After an incubation period of 7–14 days, high fever, chills, headache, rash, and an eschar usually develop in infected persons (*3*).

Scrub typhus is endemic to northern Thailand, especially Chiang Mai Province, where >200 cases are reported each year (*4*). During June 2006–May 2007, a total of 142 febrile children with clinically suspected scrub typhus were admitted to Nakornping Hospital in the city of Chiang Mai. Serologic and molecular laboratory test results showed that 65 of the children were positive for *O. tsutsugamushi*. Among the 142 hospitalized children, 30 were Hmong hill tribe people living in Ban Pongyeang, a village in the mountain area located north of the Chiang Mai. Laboratory testing also confirmed that 26 of the 30 Hmong children had scrub typhus.

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To better characterize the specific strain(s) of *O. tsutsugamushi* present in the area and to determine how the agent(s) is transmitted to humans, we genetically typed *O. tsutsugamushi* obtained from these 26 children and small mammals. The Royal Thai Army Medical Department Ethical Committee approved all procedures (protocol S014q/45). Small mammals were handled according to guidelines in the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication no. 85–23, revised 1985).

The Study

We obtained clinical information and blood samples from 26 scrub typhus–infected children from Ban Pongyeang after their parents gave informed consent. Blood specimens were stored in liquid nitrogen and shipped on dry ice to the Armed Forces Research Institute of Medical Sciences in Bangkok, Thailand, for serologic testing, genetic characterization, and isolation of *O. tsutsugamushi*.

We assessed serum samples for the presence of antibodies against *O. tsutsugamushi* by using an indirect fluorescence antibody assay (*5*) with an in-house antigen preparation from propagated *O. tsutsugamushi* Karp, Kato, and Gilliam strains. Single specimens with an IgM or IgG titer ≥400 were considered positive; paired specimens were considered positive if they showed seroconversion or a ≥4-fold rise in titer (*6*). To genetically characterize *O. tsutsugamushi*, we amplified a fragment of the 56-kDa type-specific antigen gene from patients' blood genomic DNA by using a modified nested PCR procedure as described (*7*). A newly designed forward primer (F584, 5′-CAA TGT CTG CGT TGT CGT TGC-3′) was used with the previously reported reverse primers RTS9 and RTS8 (*7*). The expected 693-bp products were purified, directly sequenced, and aligned according to ClustalW algorithm (www.clustal.org/). Using PAUP 4.0b10 software and maximum parsimony methods, we generated phylogenetic relationships (*8*). *O. tsutsugamushi* was isolated by using animal inoculation and L-929 mouse fibroblast cell culture techniques as described (*9*).

Patient clinical information and laboratory test results are shown in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/5/12-1445-Techapp1.pdf). The patients' ages ranged from 11 months to 13 years. Common signs and symptoms of illness were fever (100.0%), chills (73.1%), eschar (73.1%), headache (57.7%), and rash (23.1%) (online Technical Appendix; Figure 1). Of the 26 patients, 23 showed seroreactivity to *O. tsutsugamushi* antigens; PCR confirmed the presence of *O. tsutsugamushi* DNA in 24/26 patients (online Technical Appendix). Two *O. tsutsugamushi* isolates (PYH1 and PYH4) were successfully established from EDTA whole blood samples of 7 patients (online Technical Appendix). Patient histories revealed that the infected children commonly played in

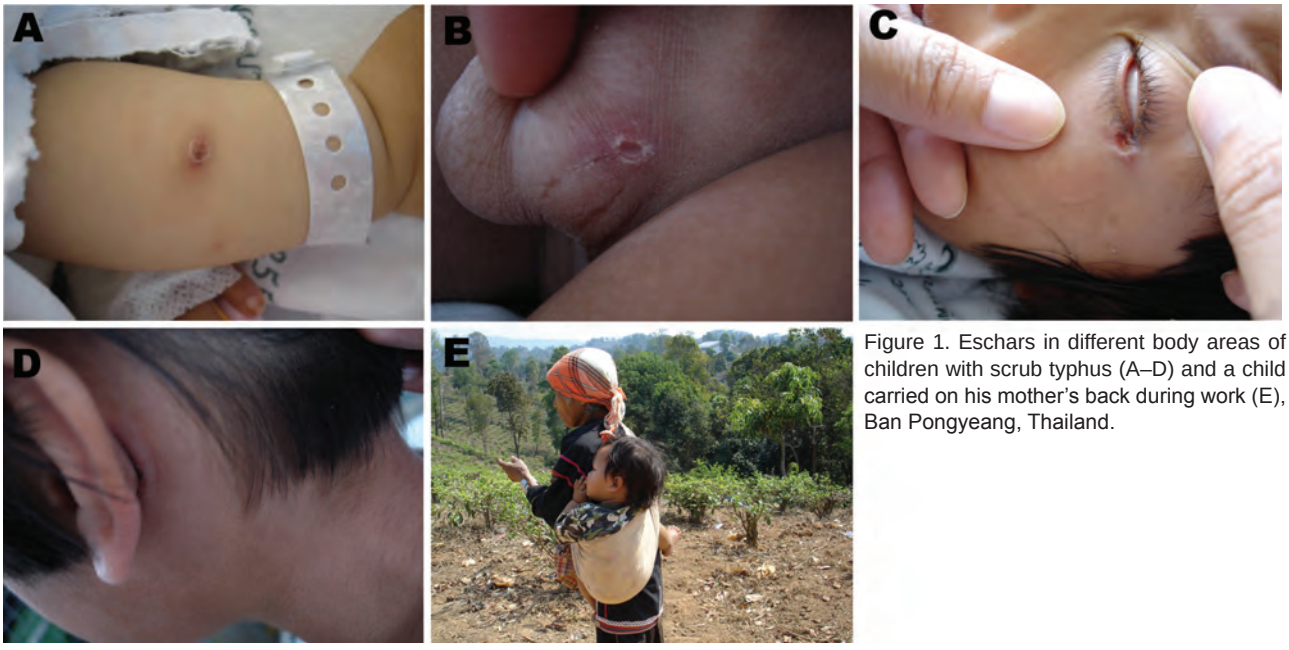

Figure 1. Eschars in different body areas of children with scrub typhus (A–D) and a child carried on his mother's back during work (E), Ban Pongyeang, Thailand.

grassland, woods, and rice fields. Cases also occurred in infants who were carried on their mother's back during work in those areas (Figure 1E). In addition, the opportunity to become infected was increased by frequent exposure to vector mites living in vegetation-rich areas.

To investigate *O. tsutsugamushi* transmission, we trapped small mammals from different terrains in Ban Pongyeang, identified them to species level, and collected tissue specimens (whole blood, liver, and spleen). The specimens were kept in liquid nitrogen and delivered to the Armed Forces Research Institute of Medical Sciences for laboratory testing. Chiggers were removed from captured mammals and stored in 70% ethanol. The chiggers were slide-mounted and identified to species by using a microscope.

A total of 55 small wild mammals were captured from different terrains in Ban Pongyeang, such as grass, rice, and banana fields and areas with shrubs and woods. The collected animals included greater bandicoot rats (*Bandicota indica*), Savile's bandicoot rats (*B. savilei*), black rats (*Rattus rattus*), small white-tooth rats (*R. berdmorei*), Polynesian rats (*R. exulans*), Berdmore's ground squirrels (*Menetes berdmorei*), a common tree shrew (*Tupaia glis*), and a small Asian mongoose (*Herpestes javanicus*) (Table 1).

Forty-five (81.8%) mammals were infested with a total of 2,277 chiggers (Table 1). A *B. indica* and a *B. savilei* rat had the highest chigger densities. Collected chiggers were classified to 4 species: *Leptotrombidium deliense* (47.6%; a well-known vector of scrub typhus), *Gahrliepia (Walchia) rustica* (35.1%), *G. (Schoengastiella) ligula* (14.6%), and *Ascoschoengastia* spp. (2.7%) (Table 2).

Thirty-six (65.5%) of 51 animals tested were seroreactive to *O. tsutsugamushi* (Table 1). Compared with the other animals, a higher percentage (100%) of *B. indica* rats had *O. tsutsugamushi* infections, indicating that this species might serve as a reservoir host for the bacterium (Table 1).

Table 1. Chigger infestation and *Orientia tsutsugamushi* infection in small mammals captured in Ban Pongyeang, northern Thailand, 2006–2007*

Rodent family, genus species	No. animals captured	No. (%) animals infested with chiggers	No. chiggers collected (mean no./animal)	No. (%) animals with *O. tsutsugamushi* infection	No. (%) *O. tsutsugamushi* isolates obtained
Muridae					
Bandicota indica	15	15 (100.0)	951 (63.4)	15 (100.0)	2 (22.2)
B. savilei	12	12 (100.0)	699 (58.2)	9 (75.0)	0
Rattus rattus	15	8 (55.6)	320 (21.3)	8 (53.3)	0
R. exulans	3	1 (50.0)	40 (5.0)	1 (33.3)	0
R. berdmorei	6	4 (66.6)	168 (28.0)	3 (50.0)	0
Viverridae, *Herpestes javanicus*	1	1 (100.0)	7 (7.0)	ND	NA
Sciuridae, *Menetes berdmorei*	2	2 (100.0)	56 (28.0)	ND	NA
Tupaiidae, *Tupaia glis*	1	1 (100.0)	41 (41.0)	ND	NA
Total	55	45 (81.8)	2,277 (44.4)	36 (65.5)	2 (3.6)

*ND, not done; NA, not applicable.

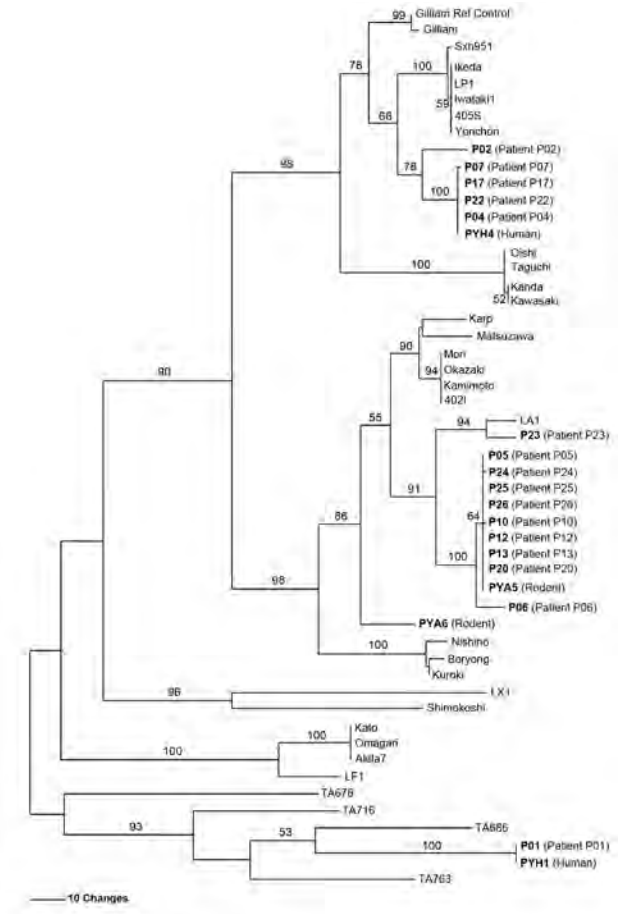

Figure 2. Maximum parsimony phylogenetic tree of *Orientia tsutsugamushi* based on partial 56-kDa type-specific antigen gene sequences, demonstrating the relationships among *O. tsutsugamushi* isolates from Thailand and strains causing scrub typhus in humans in Ban Pongyeang, Thailand, and reference (ref) strains. The tree was midpoint rooted. Bootstrap values >50% are labeled over branches (1,000 replicates). Isolates from Thailand are in **boldface**. The tree was generated by using heuristic search with random stepwise addition (10 replicates). Scale bar indicates nucleotide substitutions per site.

Because of limitations of commercial secondary antibodies, we could not perform indirect fluorescence antibody assays for the captured *T. glis* shrew (1), *M. berdmorei* ground squirrels (2), and *H. javanicus* mongoose (1). Two *O. tsutsugamushi* isolates (PYA5 and PYA6) were established from livers and spleens of 2 *B. indica* rats (Table 1). Together, the high prevalence of *O. tsutsugamushi*–seroreactive small mammals and the presence of infested scrub typhus–specific arthropod vectors indicate that scrub typhus is endemic to the Ban Pongyeang area.

O. tsutsugamushi obtained from the infected children and small mammals was characterized on the basis of *Orientia* spp.–specific 56-kDa gene fragments. Multiple alignment and phylogenetic analysis demonstrated that the 4 *O. tsutsugamushi* isolates from Ban Pongyeang fell into 4 clusters. Sequences for 3 of the isolates clustered with Gilliam, LA, and TA, 3 genotypes that are commonly found in Southeast Asia (*10*,*11*); the sequence of the fourth isolate presented as a divergent distinct genotype (Figure 2). Most of the children were infected with a strain genetically similar to the LA cluster (Figure 2). Moreover, this major pathogenic strain was recovered from *B. indica* bandicoot rats (isolate PYA5), the most commonly found rats in the village and the small mammals with the highest densities of *L. deliense* chiggers. These findings indicate possible transmission between animals and humans. Many studies have demonstrated that chiggers can acquire *O. tsutsugamushi* during the feeding process (*12*–*15*). Therefore, rodents could play a critical role as reservoir hosts for *O. tsutsugamushi* and for feeding vector mites, causing widespread distribution of *O. tsutsugamushi* in Ban Pongyeang.

Conclusions

Investigation of scrub typhus in Ban Pongyeang, northern Thailand, demonstrated *O. tsutsugamushi* infection in children and rodent hosts, and it demonstrated the potential for transmission between small mammal reservoirs and humans. Campaigns concerning protection from scrub typhus should be established in areas where *O. tsutsugamushi* is endemic, and local medical clinics should be made aware of the campaigns. Specific plans for protecting against/preventing *O. tsutsugamushi* transmission are crucially needed to prevent scrub typhus infection in humans.

Table 2. Species of chiggers collected from small mammals, Ban Pongyeang, northern Thailand, 2006–2007

Host species	No. (%) chiggers				Total
	Leptotrombidium deliense	*Gahrliepia (Walchia) rustica*	*Gahrliepia (Schoengastiella) ligula*	*Ascoschoengastia* spp.	
Bandicota indica	471 (49.5)	324 (34.1)	131 (13.8)	25 (2.6)	951
Bandicota savilei	354 (50.7)	223 (31.9)	105 (15.0)	17 (2.4)	699
Rattus rattus	125 (39.1)	119 (37.2)	56 (17.4)	20 (6.3)	320
Rattus exulans	28 (70.0)	12 (30.0)	0	0	40
Rattus berdmorei	52 (31.0)	80 (47.6)	31 (18.5)	5 (2.9)	168
Tupaia glis	15 (36.6)	17 (41.5)	9 (21.9)	0	41
Menetes berdmorei	32 (57.1)	24 (42.9)	0	0	56
Herpestes javanicus	7 (100.0)	0	0	0	7
Total	1,084 (47.6)	799 (35.1)	332 (14.6)	62 (2.7)	2,277

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Detecting *Rickettsia parkeri* Infection from Eschar Swab Specimens

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The typical clinical presentation of several spotted fever group *Rickettsia* infections includes eschars. Clinical diagnosis of the condition is usually made by analysis of blood samples. We describe a more sensitive, noninvasive means of obtaining a sample for diagnosis by using an eschar swab specimen from patients infected with *Rickettsia parkeri*.

Until 2004, all confirmed cases of tick-borne spotted fever in North, Central, and South America were attributed to 1 pathogen, *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever. Historically, in the Western Hemisphere, tick-borne rickettsiae other than *R. rickettsii* were often described as nonpathogens (*1*).

In 2004, an otherwise healthy US serviceman living in the Tidewater region of eastern Virginia, USA, sought treatment at an acute care clinic with fever, mild headache, malaise, diffuse myalgias and arthralgias, and multiple eschars on his lower extremities. He reported frequent tick and flea exposures but could not recall a specific arthropod bite before illness. However, *R. parkeri*, a tick-associated *Rickettsia* species, was subsequently isolated from an eschar biopsy specimen, documenting the first recognized case of *R. parkeri* rickettsiosis (*2*,*3*). In 2006, another US serviceman visited the National Naval Medical Center with similar symptoms. He had recently returned from a vacation in the Virginia Beach area; subsequently, *R. parkeri* was also isolated from this patient (*4*). To date, >25 cases of *R. parkeri* infections have been diagnosed in the United States and South America (*5*).

R. parkeri was first isolated from Gulf Coast ticks (*Amblyomma maculatum*) in 1937. The organism remained relatively obscure for the next several decades. *R. parkeri* is a member of the spotted fever group (SFG) of rickettsiae, which are gram-negative obligate intracellular rod-shaped bacteria transmitted by an arthropod vector. *A. maculatum* ticks, the vectors for *R. parkeri* in the United States, have a distribution that extends across all states bordering the Gulf of Mexico and includes several other southern, mid-Atlantic, and central states (*6*). *R. parkeri* has been detected in or isolated from *A. maculatum* ticks in many of these states.

Generally, the clinical signs and symptoms of SFG rickettsioses begin 6–10 days after a person has been bitten by an infected arthropod and typically include fever, headache, myalgias, a characteristic inoculation eschar at the bite site (depending on *Rickettsia* species) (Figure), a macular or maculopapular rash, and regional lymphadenopathy (*3*). SFG rickettsioses in humans range from mild to life threatening. Rocky Mountain spotted fever is considered the most severe SFG rickettsiosis; mortality rate can be as high as 50% without adequate antimicrobial drug treatment (*7*). Death from the rickettsioses can generally be prevented if diagnosis is timely and proper treatment is given.

The Study

In an ongoing, prospective study of clinical rickettsial disease in the Tidewater region of Virginia and Jacksonville, Florida, one of the primary objectives is to identify the prevalence of *R. parkeri* infection among persons seeking care with tick bite eschars or who have received a clinical diagnosis of rickettsial illness. One patient, a 43-year-old man, visited his primary care clinic in Virginia in early June 2011 after an eschar developed on his left knee, where he had removed an embedded tick 8 days previously. Topical mupirocin was prescribed for his condition. However, later that day, he experienced a fever of 104°F (40°C), accompanied by chills, night sweats, a diffuse maculopapular rash, headache, mylagias, neck stiffness, arthralgias, and malaise. He returned to the primary care clinic 3 days after onset of the fever and was prescribed a 2-week course of doxycycline. He fully recovered after the course of doxycycline.

Whole blood specimens and a swab specimen of the unroofed eschar were collected at the time he sought treatment with the acute febrile illness before doxycycline administration. Blood was collected again 25 days later during the convalescent phase. The plasma and the buffy coat were separated from the erythrocytes after centrifugation. The eschar swab was rinsed in 300 μL of phosphate-buffered saline. DNA was extracted from the buffy coat and the swab sample rinse by using QIAamp blood mini kit (QIAGEN, Germantown, MD, USA). Three microliters of the DNA preparations were applied to the *Rickettsia* genus–specific quantitative real-time PCRs (qPCRs) targeting the 17-kDa antigen gene (Rick17b) (*8*) and *gltA* (*9*), as well as the *R. parkeri* species–specific qPCR (Rpark), as described (*8*). Positive reactions were obtained from all 3 qPCRs for the swab sample and the Rick17b assay for the buffy coat.

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Thus, the diagnosis of *R. parkeri* infection in this patient was confirmed by molecular assays. The *R. rickettsia*–specific qPCR (Rrick) (*10*) yielded negative results.

Acute-phase and convalescent-phase plasma samples were tested for SFG-specific IgG and IgM by ELISA using *Rickettsia conorii* whole cell antigen preparation (*11*). The ELISA was performed side by side in 4-fold dilutions from 1:100 to 1:6,400. The SFG group–specific IgM was not detected in both samples, and the SFG group–specific IgG was also not detected in the acute sample but was detected in the convalescent-phase sample at a titer of 400. The serologic results confirmed the patient's infection with a SFG rickettsial agent.

A second patient, a 36-year-old man, sought treatment at a primary care clinic in Pensacola, Florida, in late August 2011, 10 days after he was bitten by a tick. He was otherwise healthy, but a painless eschar had developed on his left ankle ≈4 days after exposure to the tick. Over the next several days, a generalized vesicular rash developed on his torso and upper and lower extremities, along with fevers >101°F, chills, night sweats, a mild headache, and generalized lymphadenopathy. The patient was prescribed a 14-day course of doxycycline at his initial visit. His symptoms improved within 24 hours. Serum samples were collected at his initial clinic visit, before initiation of antimicrobial drug therapy.

He returned to the clinic and was found to be asymptomatic after 14 days of treatment. Whole blood and a swab of his healing eschar–the crust was unroofed on sampling–were taken at his return visit. DNA was extracted from acute-phase serum and the eschar swab by using the same procedure as had been used for the first patient. Positive reactions were obtained from the swab sample by Rick17b and Rpark qPCRs, and negative by Rrick assay. The acute-phase serum sample was negative by all qPCRs mentioned above. The acute-phase serum and the convalescent-phase plasma samples were tested for SFG rickettsiae–specific IgM and IgG as was performed for the first patient. The titer of IgM for both samples was 400, whereas the IgG for the acute-phase serum and the convalescent-phase plasma samples were 1,600 and 6,400, respectively. The 4-fold raise in IgG titer between acute- and convalescent-phase samples provided the evidence for a current infection.

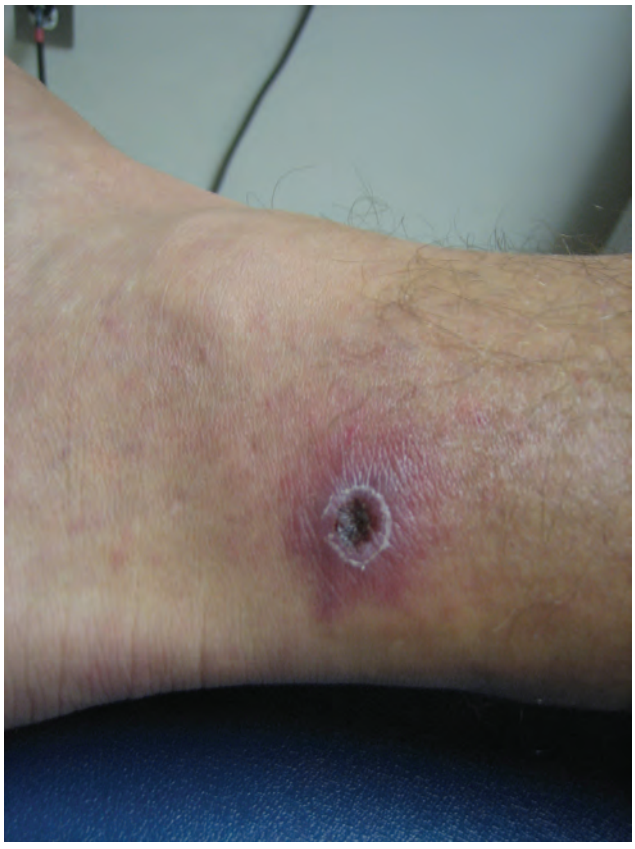

Figure. Acute eschar of patient who was subsequently diagnosed with *Rickettsia parkeri* infection in Pensacola, Florida, USA, in August 2011. This same eschar was unsheathed and swabbed after 14 days of antimicrobial drug treatment and had undergone significant healing. It still gave a positive result by real-time PCR, although the convalescent-phase blood specimen showed a negative result.

Conclusions

Although *R. rickettsii* is reportedly the predominant pathogen causing human SFG disease in the United States, the actual prevalence of *R. parkeri* is unknown because most commercial serologic assays do not distinguish between SFG species. Seroprevalence studies have also demonstrated higher than expected *R. rickettsii* seropositivity among the general population without history of Rocky Mountain spotted fever, which suggests that they may have been infected by less pathogenic SFG rickettsiae such as *R. parkeri*. Recently, qPCRs have demonstrated to be effective at identifying rickettsiae specific to the species level (*12*). A report by Bechah et al. (*12*) has shown that rickettsial infection could be diagnosed from noninvasively collected cutaneous lesion swab specimens from skin eschars from guinea pigs.

Using this model and our qPCR Rick17b (*8*), we were able to obtain positive results not only from the buffy coat sample from the Tidewater patient but also a positive reaction from a swab specimen from the eschar of the same patient. In the second patient, we were able to obtain a positive reaction from the swab specimen from the healing eschar 14 days after antimicrobial drug treatment. This finding indicates that positive reaction results may be obtained from a healing eschar 14 days after antimicrobial drug treatment and is consistent with the recent report of diagnosis of SFG from swab specimens from patients in Algeria (*13*). In addition, evidence is mounting that eschars can be tested for rickettsial DNA from the point of signs and symptoms all the through the convalescent phase.

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Dr Myers is a Public Health Service officer and the clinical laboratory director for the Naval Infectious Diseases Diagnostic Laboratory. His research interests include clinical diagnostic assay development, medical microbiology, and molecular diagnostics.

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Contaminated Ventilator Air Flow Sensor Linked to *Bacillus cereus* Colonization of Newborns

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We investigated *Bacillus cereus*–positive tracheal aspirates from infants on ventilators in a neonatal intensive care unit. Multilocus sequence typing determined a genetic match between strains isolated from samples from a case-patient and from the air flow sensor in the ventilator. Changing the sterilization method for sensors to steam autoclaving stopped transmission.

Because of ubiquity in the environment, the recovery of *Bacillus* species from clinical specimens is often considered a clinically inconsequential contamination. Nevertheless, an accumulating body of literature suggests that contamination with this organism should not be routinely dismissed (*1*). Severe and lethal *Bacillus cereus* infections have been described in newborn infants, with higher frequency among premature infants. The types of *B. cereus* infections in newborns included central nervous system, respiratory tract, primary bacteremia, and sepsis (*2*–*4*). Nosocomial outbreaks of *B. cereus* implicating hospital linens, manual ventilation balloons, contaminated diapers, and contaminated ventilator equipment have also been reported (*5*–*9*).

The Study

The Missouri Department of Health and Senior Services conducted this investigation in response to the hospital's identification of an increased number of tracheal aspirates that were positive for *B. cereus* collected from newborns who were on ventilators during March–May, 2011. All tracheal aspirate culture results obtained in the Neonatal Intensive Care Unit (NICU) during January 2010–June 2011 were reviewed. NICU data was also searched for positive *B. cereus* culture from other specimens, such as blood, body fluids, or tissues. Investigators thoroughly evaluated respiratory management practices in the unit by direct observation, respiratory records review, and an interview with the respiratory therapist.

Several environmental cultures were obtained from the flow sensors of the unit's ventilators over the 1-month period. *B. cereus* isolates were forwarded to the Centers for Disease Control and Prevention to be molecularly characterized by using multilocus sequence typing (MLST) (*10*). DNA was prepared from bacterial cultures as described (*11*). The DNA was used as a template in PCRs with the primers described on the *Bacillus cereus* MLST Web site (www.pubmlst.org/bcereus) for the 7 loci which define the MLST scheme. The sequences for the loci *glpF*, *gmk*, *ilvD*, *pta*, *pur*, *pycA*, and *tpi* were then assigned allele designations. The combination of the 7 alleles determines a given sequence type. A greater number of alleles that match between strains indicates a higher level of relatedness (*10*). Prevalence of *B. cereus*–positive specimens was compared by using the Mann-Whitney U test.

Retrospective analysis of tracheal aspirate culture results showed significant increase ($p = 0.039$) in B. cereus isolation between March and May, 2011 (Figure 1). No Bacillus spp. were isolated from blood, other body fluids, or tissues during the study period. The chart review of the case-patients comprising the cluster of B. cereus colonization revealed that none received a diagnosis of clinical B. cereus infection. All patients were treated with vancomycin or tobramycin, or both, for indications not related to B. cereus in tracheal aspirate. One case-patient died 108 days later without evidence that B. cereus contributed to the outcome. All other case-patients recovered and were discharged.

Investigation of the ventilation procedures in the NICU revealed that most equipment used for respiratory care was disposable, designated for single-patient use. The Draeger Evita v500 ventilator (Draeger Medical Inc., Telford, PA, USA; www.draeger.us/sites/enus_us/pages/hospital/evita-xl.aspx) was used for mechanical ventilation of infants who were intubated to treat severe respiratory compromise. The Draeger Evita V500 is a microprocessor controlled ventilator offering both mandatory and spontaneous ventilation modes for adult, pediatric, and neonatal patients. Heated and humidified gas flows from the ventilator unit, through the inspiratory circuit and NeoFlow air flow sensor to the patient through an endotracheal tube. Upon exhalation, gas flows back through the air flow sensor into the expiratory circuit and returns to the ventilator through the expiratory flow sensor and exhalation valve. In addition

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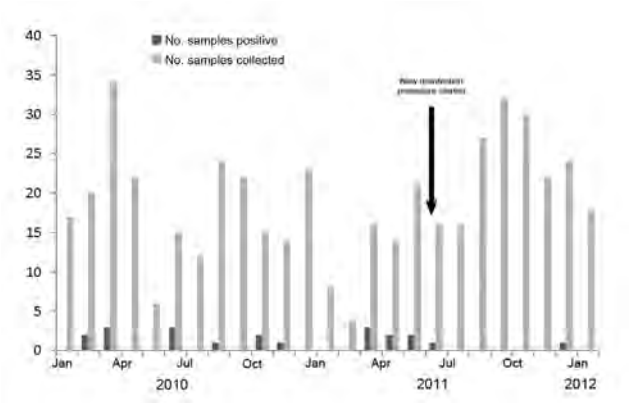

Figure 1. Epidemiologic curve of *Bacillus* spp.–positive tracheal aspirates from newborns on ventilators, January 2010–January 2012.

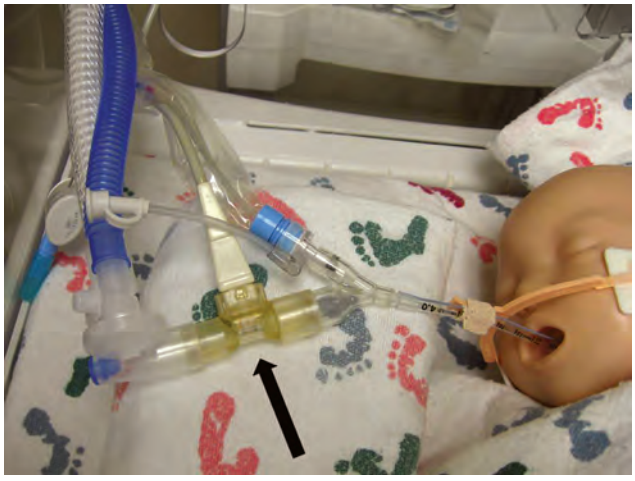

Figure 2. Draeger Evita v500 respirator. Arrow indicates Neoflow air flow sensor.

to the ventilator, reusable respiratory equipment comprised a proximal air flow sensor, expiratory flow sensor, exhalation valve, and circuit temperature probe. The sensor closest to the newborn's mouth was an air flow sensor located inside the disposable ventilation circuit (Figure 2). From 9 environmental cultures obtained from 9 air flow sensors, 1 was positive for *Bacillus* spp., and was later confirmed as *B. cereus* by the State Public Health Laboratory.

MLST was performed for 8 *B. cereus* isolates from case-patients and for 1 environmental isolate from the air flow sensor. We were able to fully characterize 4 of the 9 isolates (Table). One locus for the remaining 5 strains did not yield an amplicon for sequencing after repeated attempts and, thus, could not be assigned a sequence type. The isolates that included sequence type (ST) 73 and ST94 were closely related to each other because they differed by merely 1 locus, *gmk*. The strains that were not fully typed because of the inability to obtain sequences for locus *pta* were also closely related to ST73 or ST94 because the other loci matched. There was 1 match between strains isolated from 1 case-patient and the air flow sensor, which was ST73. The contaminated air flow sensor was then sterilized by using a steam autoclave. A repeat culture of this sensor after sterilization was negative.

We found that air flow sensors were routinely disinfected by placing them in a container with 70% alcohol solution for 60 minutes. After discovery of the air flow sensor contaminated with *B. cereus*, the disinfection policy was changed. All air flow sensors were first soaked in Enzol enzymatic detergent (ASP, Irvine, CA, USA; www.aspjj.com/us/products/enzol) solution and then sent for steam autoclave sterilization at 134°C (273.2°F). After implementation of new disinfection and sterilization procedures, no new cases of *B. cereus* tracheal colonization were identified in the nursery. In this cluster, contaminated proximal air flow sensors were the likely source of tracheal colonization with *B. cereus* in newborn infants, supported by a genetic match by MLST between a strain isolated from 1 case-patient and the contaminated air flow sensor.

Conclusions

B. cereus transmission from contaminated respiratory equipment has been reported in other geographic areas. In the Netherlands, an outbreak of *B. cereus* infections in a pediatric intensive care unit caused by contaminated reusable ventilator air flow sensors was described (*7*). Switching to disposable air flow sensors stopped colonization with

Table. Alleles and sequence types determined for *B. cereus* isolates associated with contaminated ventilator air flow sensor linked to colonization of newborns

	Alleles							
Isolate origin	*glpF*	*gmk*	*ilvD*	*pta*	*pur*	*pycA*	*tpi*	Sequence type*
Patient 1	13	29	9	Null	9	12	31	ND
Patient 1	13	8	9	Null	9	12	31	ND
Patient 2	13	29	9	14	9	12	31	94
Patient 3	13	8	9	14	9	12	31	73
Patient 4	13	29	9	14	9	12	31	94
Patient 5	13	29	9	Null	9	12	31	ND
Patient 6	13	8	9	Null	9	12	31	ND
Patient 7	13	29	9	Null	9	12	31	ND
Airflow sensor	13	8	9	14	9	12	31	73

*ND, not determined.

B. cereus in that unit. In Canada, an outbreak of *B. cereus* infections among patients in an adult ICU was linked to colonized ventilator circuitry (*8*). In the United Kingdom, reusable ventilator circuits were also identified as the cause of a *B. cereus* outbreak among intubated NICU patients (*9*).

Our investigation underscores the necessity of close monitoring of occurrences of *Bacillus* spp. in tracheal aspirates since clustering of such cases could be an indication of single source contamination. In our investigation, *B. cereus* isolates were either ST73, ST94, or closely related to those sequence types. ST73 and ST94 are associated with strains previously described as having caused illness in elderly persons. Strains with ST73 were implicated in cases of septicemia (*12*), and of sepsis and pneumonia (*13*). Strains with ST 94 were recovered from patients with pneumonia (*14*). *B. cereus* strains harboring *B. anthracis* plasmids such as pXO1, have also been associated with severe and fatal respiratory infections (*15*).

All case-patients in our investigation were considered to be colonized with *B. cereus* without clinical implications. Since all of them received intravenous antimicrobial drugs effective against *B. cereus*, it is conceivable that the clinical course of those patients could have been different without such treatment.

Bacillus spp. in tracheal aspirate cultures should not be routinely viewed as clinically insignificant and further testing to determine exact strain should be considered under appropriate clinical and epidemiologic circumstances. Proper disinfection of the entire ventilator circuit as recommended by the equipment manufacturer is crucial in avoiding potentially lethal *B. cereus* infections.

Acknowledgments

This study made use of the *Bacillus cereus* Multi Locus Sequence Typing website (http://pubmlst.org/bcereus/) developed by Keith Jolley and sited at the University of Oxford. The development of that site was funded by the Wellcome Trust.

Dr Turabelidze is a Missouri State Epidemiologist. His main research interests include epidemiology of communicable diseases.

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Mapping Environmental Suitability for Malaria Transmission, Greece

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During 2009–2012, Greece experienced a resurgence of domestic malaria transmission. To help guide malaria response efforts, we used spatial modeling to characterize environmental signatures of areas suitable for transmission. Nonlinear discriminant analysis indicated that sea-level altitude and land-surface temperature parameters are predictive in this regard.

Malaria was eliminated in Greece in 1974 (*1*,*2*); however, cases continue to be imported from countries to which malaria is endemic (*3*) and locally acquired cases have occurred sporadically (*4*,*5*). During 2009–2012, health authorities in Greece recorded 267 malaria cases. Although most cases were imported, at least 69 (26%) occurred in patients who did not have travel histories to malaria-endemic regions. A cluster of 6 locally acquired *Plamosdium vivax* malaria cases occurred during August–October 2009 in the southern Peloponnese (Evrotas Municipality, Lakonia district); in addition, 1 autochthonous case was reported from Marathon Municipality, East Attiki district (*2*). In 2010, locally transmitted cases were recorded in the same Lakonia district, 1 in East Attiki and 2 in children in central Greece (Viotia district). In 2011, a total of 42 autochthonous cases of *P. vivax* malaria were reported, representing 44% of the 96 notified cases in 2011. Most (36) of those cases were notified in the Evrotas municipality. In 2012, locally acquired cases appeared to have decreased, with 16 cases representing 21% of the overall number of cases. The ongoing transmission of *P. vivax* by local anopheline mosquitoes raises concern about reemergence of malaria transmission in Greece in areas that are hospitable to the vector and have permissive environmental and climatic conditions (*6*).

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The Study

To guide malaria control efforts, we delineated areas suitable for malaria transmission in Greece using the place of exposure for 69 locally acquired malaria cases. A health officer administered a standardized questionnaire to each malaria case-patient in Greece to determine the origin of infection. Our analysis was restricted to cases classified as locally acquired in persons without travel histories to optimize the specificity of the model because our goal was to describe the environmental suitability of autochthonous malaria transmission in Greece. Thus, our analysis excluded cases that might have been acquired abroad.

We aimed to describe the environmental profile of areas with active transmission cycles during 2009–2012 and then to predict other areas at risk for malaria reemergence in Greece. Our data sources were countrywide georeferenced environmental and climatic information, all acquired from the European Environment and Epidemiology Network data repository and prepared for spatial modeling (*7*). Variables considered were temperature, vegetation seasonal variations, altitude, land-cover categories, and demographic indicators. Daytime and nighttime Land Surface Temperature and Normalized Difference Vegetation Index were retrieved from the 1 km–resolution long-term Temporal Fourier transformed imagery from Moderate Resolution Imaging Spectroradiometer (*8*). Altitude values were derived from the Global Land One-km Base Elevation Digital Elevation Model data from the US Geological Survey Earth Resources Observation Systems data center (http://eros.usgs.gov). The coordination of information on the environment (CORINE) land cover from the European Environment Agency provided the framework for land cover patterns exploratory analysis (www.eea.europa.eu/data-and-maps/data/corine-land-cover-2000-clc2000-seamless-vector-database-3). The population density grid of the Joint Research Centre was used as demographic indicator (www.eea.europa.eu/data-and-maps/data/population-density-disaggregated-with-corine-land-cover-2000-1).

The spatial scale for this study was a polygon equivalent to a circular buffer of 3.5 km (40,000 ha). This spatial resolution was considered appropriate to the relatively fine scale at which environmental variability can affect malaria transmission. The construction of the land-cover variables is described in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/5/12-0811-Techapp1.pdf). A disease risk map was generated by using nonlinear discriminant analysis available in eRiskMapper version 1.1.4 (www.tala.ox.ac.uk) and is described in detail in the online Technical Appendix.

Table. Environmental suitability mapping of malaria, Greece, 2009–2012*

Variable†	Rank
Digital elevation model	4.4
LST day amplitude 2	5.5
NDVI phase 2	7.0
LST nighttime amplitude 2	7.3
LST nighttime mean	8.1
LST day, maximum	8.1
LST day, mean	8.3
LST day phase 2	8.6
NDVI, maximum	8.7
LST night, maximum	8.9

*Classification of 10 variables average rank (1 to 10) for all bootstrapped cycles. Data sources are as follows: Global 30 Arc-Second Elevation Dataset (GTOPO30), 2005 (http://eros.usgs.gov); CORINE Land Cover 2000 seamless vector data, version 15 (Aug 2011), European Environment Agency, 2011 (www.eea.europa.eu/data-and-maps/data/corine-land-cover-2000-clc2000-seamless-vector-database-3); Raster data on population density using CORINE Land Cover 2000 inventory, European Environment Agency. 2009 (www.eea.europa.eu/data-and-maps/data/population-density-disaggregated-with-corine-land-cover-2000-2).
†LST, Land Surface Temperature; NDVI, Normalized Difference Vegetation Index.

The first 10 best ranked variables comprised sea-level altitude and land-surface temperature (Table). Parameters of nighttime and daytime temperatures were predictive in this model; the annual variation (amplitude) and the mean absolute temperature values scored high in the ranking. Predicted suitability of areas for persistent malaria transmission based on these variables are characterized by low elevation; warmer temperatures (which might enable more rapid mosquito and parasite development); and intensive, year-round irrigated agriculture with complex cultivation patterns (generally requiring a high degree of manual labor) (Figure 1). These are probable contributing factors to mosquitoe presence and, possibly, to malaria transmission.

The accuracy statistics of the bootstrap model revealed a sensitivity of 0.98 (where 1 denotes recognition of all actual presences) and specificity of 0.98 (where 1 denotes recognition of all actual absences). Moreover, historical presence of malaria in Greece, before disease elimination, was extracted from several sources and showed a partially coincident distribution pattern with the suitability map: 41% overlap in Peloponnese, 63% along the west coast of central Greece and Epirus, and 39% in eastern central Greece (Figure 2). The best overlap was found along the west coast, but in the northern part, the model did not match the historical pattern very well. This discrepancy might be explained by the low rank of landscape pattern variables obtained into nonlinear discriminant analysis modeling.

Conclusions

We assessed environmental and climatic characteristics of the areas with autochthonous spread of *P. vivax* malaria in Greece during 2009–2012 and delineated similar areas possibly suitable for transmission in Greece. Sea-level altitude and the mean and annual variation of land-surface temperature for daytime and nighttime were predictors in our model.

A major limitation of our study is that it considers only environmental suitability for transmission, not risk for transmission per se. Our approach did not account for certain factors in this regard, for example, presence/absence of malaria vectors under collection in 2012. This partly reflects the lack of knowledge about what mosquito species were responsible for recent outbreaks, although historic work suggests *Anopheles sacharovi*, *An. maculipennis s.s.*, and *An. superpictus* (*9*,*10*). Indeed, 2 of these species, *An. sacharovi* and *An. superpictus*, have been implicated as the probable dominant vectors throughout Greece and adjacent areas (*11*). Hence, species would seem to be reasonable targets for any mosquito surveillance efforts.

Our model did not highlight areas of northern Greece previously associated with malaria (*4*,*5*) as being particularly suitable for transmission. Further refinement of our approach, perhaps including vector information, might address this shortcoming. Further study on the malariogenic potential should be conducted to complete our assessment. Despite these limitations, this spatial analysis can be useful and can help guide the public health response to threats, such as malaria, by directing surveillance and control activities and/or by identifying uncertainties relevant to disease risk mapping and response.

Model accuracy and public health practice can be improved through vigilant disease and vector surveillance with timely case notification (*12*). Yet, despite these potential limitations, we believe that this spatial analysis is a useful tool; it can help guide response(s), integrated preparedness and response activities, including targeted epidemiologic and entomologic surveillance, vector control

Figure 1. Areas latently hospitable and environmentally permissive for persistent malaria transmission, Greece, 2009–2012. Map showing areas predicted to be environmentally suitable for malaria transmission. Values from 0 to 0.5 (dark to light green) indicate conditions not favorable for malaria transmission (based on locally acquired cases); yellow to dark red areas delineate conditions increasingly favorable for transmission (values from 0.5 to 1).

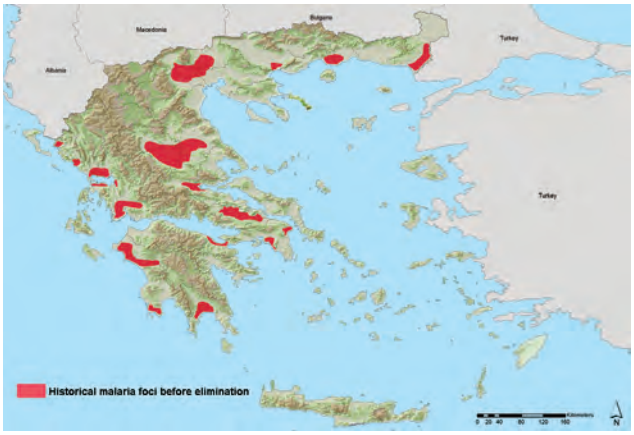

Figure 2. Areas of historic malaria transmission before elimination, Greece. Greece was officially declared malaria free in 1974, after a national malaria elimination effort during 1946–1960. Data sources: adapted from (*10*); Ministry of Health. Map of confirmed laboratory species–1952, unpub. data.

activities, and awareness rising among the general population and health care workers, in the areas environmentally suitable for transmission.

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Implications of Dengue Outbreaks for Blood Supply, Australia

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Dengue outbreaks have increased in size and frequency in Australia, and transfusion-transmitted dengue poses a risk to transfusion safety. Using whole blood samples collected during the large 2008–2009 dengue epidemic, we estimated the risk for a dengue-infectious blood donation as ≈1 in 7,146 (range 2,218–50,021).

Dengue causes >50 million infections per year worldwide; however, the true incidence is expected to be higher given that asymptomatic infection is possible (*1*). Dengue virus types 1–4 (DENV-1–4) are emerging or reemerging in many regions of the world (*1*,*2*), including Australia (*3*). One of the largest epidemics in at least 50 years occurred in Queensland, Australia, during 2008–2009, with separate outbreaks in Cairns (and surrounding regions; DENV-2, DENV-3; 2008–2009), Innisfail (DENV-4; 2009), and Townsville (DENV-1, DENV-3; 2009), totaling >1,000 confirmed clinical cases (*3*).

Infection with DENVs poses a risk for transfusion safety, and 5 cases of transfusion-transmitted dengue have been reported (*4*,*5*). In addition, DENV RNA has been detected in asymptomatic blood donors from areas to which dengue is endemic (*6*–*8*). Given the absence of an approved blood screening test for dengue in Australia, managing transfusion-transmission risk focuses on identifying donors at risk for exposure and temporarily excluding them from donating fresh blood components (erythrocytes, platelets, and clinical plasma) (referred to here as dengue management strategy) (*9*). Plasma collection for fractionation can continue because the process of manufacturing concentrates inactivates the virus (*10*). This approach assists with meeting an expanding demand for intravenous Ig but may result in fresh component losses and be associated with considerable cost.

Risk to the blood supply correlates with asymptomatic donor viremia; understanding the rate of dengue subclinical infection in countries to which it is not endemic and local northern Queensland seroprevalence is necessary for assessing this risk. We examined dengue seroprevalence rates in Australian donors during this epidemic; used these data to estimate the subclinical infection rate, population prevalence, and associated transfusion-transmission risk; and estimated the economic effect of this epidemic to the Australian Red Cross Blood Service (Blood Service).

The Study

Whole blood samples collected during the 2008–2009 dengue epidemic were tested for DENV IgM by ELISA (Dengue IgM Capture ELISA; Panbio, Brisbane, QLD, Australia). All reactive samples were tested with a second ELISA (Anti-Dengue IgM ELISA; Standard Diagnostics Inc., Giheung-gu, South Korea) and by the Public Health Virology Laboratory at Queensland Health Forensic and Scientific Services (QHFSS) (*11*). Serologic evidence of recent exposure (presence of DENV IgM) was observed in 12 (0.22%) donors (Table 1). Of the 8 DENV IgM–positive samples that were examined for type specificity, 7 (88%) were DENV-3 specific, which was the dominant type during the epidemic (*3*).

We used these IgM seroprevalence rates to estimate the rate of subclinical dengue infection (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1664-Techapp1.pdf). We estimated 168–921 subclinical cases (clinical:subclinical ratio 1.0:0.59; range 0.18–1.0) in Cairns, the city where the epidemic was centered. Our estimate was toward the lower end of that observed in dengue-endemic areas (*12*,*13*) but higher than that estimated during a DENV-2 outbreak in Charters Towers (*14*), which probably reflects the different methods used in the respective studies.

Selected samples were tested for DENV IgG by ELISA (Dengue IgG Indirect ELISA; Panbio). All reactive samples were tested at QHFSS. Serologic evidence of previous exposure (presence of DENV IgG) was influenced overall by donor location ($p<0.05$) and age ($p<0.05$). The proportion of the northern Queensland donor population with DENV IgG was 9.43% (95% CI 7.98%–10.89%), and this proportion increased with age (Table 2), which indicates cumulative previous exposure. The proportion of Melbourne (control area with no dengue activity) donors with DENV IgG was 6.78% (95% CI 4.48%–9.09%); however,

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Table 1. Serologic evidence of recent dengue exposure by dengue virus IgM in blood donors, northern Queensland, Australia, 2008–2009 epidemic

Location	Donors		Donations	
	No. samples	Reactive samples, no. (% [95% CI])	No. samples	Reactive samples, no. (% [95% CI])
North Queensland	5,453	12 (0.22 [0.10–0.34])	10,026	13 (0.13 [0.06–0.20])
Cairns	2,416	8 (0.33 [0.10–0.56])	5,051	9 (0.18 [0.06–0.29])
Townsville	3,037	4 (0.13 [0.00–0.26])	4,975	4 (0.08 [0.00–0.16])

no change was observed with age (Table 2), demonstrating no cumulative exposure. Previous exposure in Melbourne was surprisingly high; these persons may have been exposed during travel to dengue-endemic areas (subsequent follow-up demonstrated 94% reported travel to countries to which dengue may be endemic).

The proportion of donors with DENV IgG did not change from the beginning to the end of the outbreaks in Cairns and Townsville, nor in northern Queensland as a whole (Table 2), which suggests that the epidemic was not of a scale to result in a change in population seroprevalence. This study was powered to detect a change in incidence of at least 10%; small changes might have been missed, which would be difficult to detect through such studies.

We used our seroprevalence data along with donation frequencies to estimate the risk of collecting a dengue-infectious donation, based on published models (*9*,*15*) (online Technical Appendix). Using this model, the risk of collecting a dengue-infectious donation in Cairns during the epidemic was ≈1 in 7,146 (range 2,218–50,021) (Figure). These estimates are similar to those obtained by using a published probabilistic model (*9*) revised to incorporate the outbreak specific subclinical infection rate reported herein, which predicts the risk to be ≈1 in 9,303 (range 3,092–32,344) donations in Cairns. Because both methods derive estimates within comparable ranges, it would appear valid to use the revised probabilistic model as a predictive risk estimator during future outbreaks.

The dengue management strategy used during the epidemic cost the Blood Service ≈1–3.8 million Australian dollars (2009 terms). This estimate is publically available and was based on: the number of donations affected by the dengue management strategy, collection targets for 2009, costs associated with whole blood collections, additional costs to meet national targets, transportation costs to meet demand in affected regions, and additional waste costs. An offset for any plasma obtained through a whole blood donation (used for fractionation) was included in selected estimates.

Table 2. Prevalence of dengue virus IgG in the blood donor population, northern Queensland, Australia

Variable	No. samples	Reactive, no. (% [95% CI])
Northern Queensland	1,548	146 (9.43 [7.98–10.89])
Age group, y		
<40	567	18 (3.17 [1.73–4.62])
40–60	742	70 (9.43 [7.33–11.54])
>60	239	58 (24.27 [18.83–29.70])
Outbreak		
Start	788	72 (9.14 [7.13–11.15])
End	760	74 (9.74 [7.63–11.84])
Cairns	738	53 (7.18 [5.32–9.04])
Age group, y		
<40	271	5 (1.85 [0.24–3.45])
40–60	360	26 (7.22 [4.55–9.90])
>60	107	22 (20.56 [12.90–28.22])
Outbreak		
Start	384	31 (8.07 [5.35–10.80])
End	354	22 (6.21 [3.70–8.73])
Townsville	810	93 (11.48 [9.29–13.68])
Age group, y		
<40	296	13 (4.39 [2.06–6.73])
40–60	382	44 (11.52 [8.32–14.72])
>60	132	36 (27.27 [19.68–34.87])
Outbreak		
Start	404	41 (10.15 [7.20–13.09])
End	406	52 (12.81 [9.5–16.066])
Melbourne	457	31 (6.78 [4.48–9.09])
Age group, y		
<40	151	11 (7.28 [3.14–11.43])
40–60	215	16 (7.44 [3.93–10.95])
>60	91	4 (4.40 [0.18–8.61])

Conclusions

Subclinical dengue infection rates vary by population, specific outbreak, and area examined (*12*,*14*). We demonstrate that the clinical to subclinical infection rate during the 2008–2009 dengue epidemic in northern Queensland, where dengue occurs seasonally, was toward the lower end of that observed in dengue-endemic countries (*12*,*13*). This observation, together with our data suggesting that the incidence of dengue in the northern Queensland population did not change from the beginning to the end of the epidemic, suggests the control and clinical management of dengue during this epidemic was comprehensive.

We also estimated that the risk of collecting a dengue-infectious blood donation in Cairns during the epidemic was ≈1 in 7,146 (range 2,218–50,021). Given these risks, the increasing need for plasma in Australia, and the absence of a screening test for blood donations in Australia, the continuation of the dengue management strategy during future outbreaks is warranted. However, this strategy may have added strain on the inventory available to meet clinical demand for fresh blood components and was associated with a cost to the Blood Service of >1 million Australian dollars. Although this strategy is a necessary precaution to maintain safety, alternative approaches may exist, such as implementation of a suitable screening test (were one available) or pathogen reduction technology (a process designed to inactivate

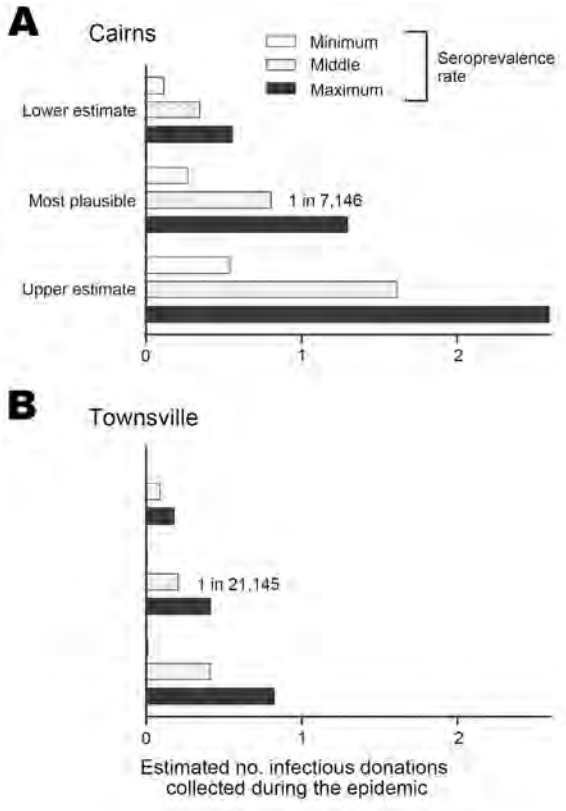

Figure. Risk of collecting a dengue-infectious blood donation, northern Queensland, Australia, 2008–2009 epidemic. Estimated risk calculated for Cairns (A) and Townsville (B).

pathogens in blood products), which may offer a similar level of safety but be more cost effective. With dengue becoming increasingly common in Australia (*3*) and the world (*1*), these alternative approaches may be needed in the future.

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Dr Faddy is a research fellow in the Research and Development Division of the Australian Red Cross Blood Service. Her current research interests and activities focus on providing an evidence base to enable evaluation of current emerging infectious risks to the safety of the Australian blood supply.

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Novel Molecular Type of *Clostridium difficile* in Neonatal Pigs, Western Australia

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Clostridium difficile causes neonatal enteritis in piglets; strains of PCR ribotype 078 are most commonly identified. We investigated *C. difficile* prevalence in piglets in Australia and isolated a novel strain with a unique pathogenicity locus. In a mouse infection model, this strain produced more weight loss than did a ribotype 078 strain.

Clostridium difficile is the causative agent of severe enteritis ("scouring") in neonatal piglets 1–7 days of age throughout Canada, the United States, and Europe (*1*). Although deaths attributable to *C. difficile* infection (CDI) generally are low because of good stockmanship, piglets that survive CDI remain 10%–15% underweight on average and take additional time to wean (*2*).

Colonization frequency of *C. difficile* in scouring piglets is as high as 52%; this rate declines to 4% by 2 months of age (*3*). *C. difficile* is also commonly found in feces from apparently healthy piglets, which contributes to environmental contamination. Widespread air and surface contamination of the piggery environment with *C. difficile*, presumably in the form of long-surviving spores, may play a role in the epidemiology of CDI in pigs and subsequent community-acquired infection in humans (*4*).

In Europe and the United States, the genotypes of *C. difficile* isolates that cause disease in humans and production animals overlap, particularly PCR ribotype 078, which predominates in pigs worldwide. This ribotype is increasing in prevalence and associated with severe community-acquired CDI in humans geographically located near pig farms (*5*). *C. difficile* has also been found in retail food, including meat products, seafood, and vegetables (*6*).

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C. difficile in piglets in Australia has not been systematically investigated, despite reports of idiopathic enteritis nationwide. It is likely that that *C. difficile* strains in piglets in Australia are different from those found in the rest of the world because of Australia's geographic isolation, strict quarantine laws regarding importation of livestock, and low human population and pig density. We studied *C. difficile* prevalence in scouring neonatal piglets and evaluated a novel strain of *C. difficile* isolated from these piglets by using multiple identification methods.

The Study

Rectal swab specimens were collected during July–November 2009 from 185 neonatal piglets on 3 farms that were experiencing scouring problems. The farms were located at 2 geographic locations in Western Australia (20 km apart) and were owned by a commercial farrow-to-finish operation. At the time of the study, 50%–80% of litters were scouring, with death rates of 11%–14%. The sick piglets had early-onset, nonhemorrhagic, yellow, pasty-to-watery diarrhea; disease course without treatment was ill-thrift, anorexia, dehydration, and death. Healthy piglets were treated prophylactically at 1–3 days of age with amoxicillin or penicillin.

Of the 185 piglets, 131 were on 2 farms at the same geographic location that had the most severe scouring problems. The remaining 54 piglets were on a high biosecurity farm at a separate location with a variable scouring problem; 11 of these animals were asymptomatic. Test results for *Escherichia coli*, rotavirus, and *C. perfringens* were negative for all animals; we did not test for porcine reproductive and respiratory syndrome virus or transmissible gastroenteritis coronavirus because they are considered exotic (i.e., no reported outbreaks) in Australia.

Samples were cultured directly onto cycloserine cefoxitin fructose agar and incubated anaerobically at 37°C for 48 h. The swabs were then inoculated into a Robertson's cooked-meat selective enrichment broth and incubated anaerobically at 37°C for 7 days; spores were then selected by alcohol shock (1:1 with anhydrous ethanol). The spores were then cultured onto cycloserine cefoxitin fructose agar with 0.1% taurocholic acid added. Putative *C. difficile* colonies were subcultured onto prereduced blood agar and identified by Gram stain, characteristic colony morphology, and smell.

Toxin profiling was by PCR detection of the toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdt*) genes (*7*,*8*). Isolates underwent PCR ribotyping and were compared with human reference *C. difficile* strains from Australia and international ribotypes from the Anaerobe Reference Laboratory (Cardiff, Wales, UK) (*9*). Genome shotgun sequencing of a representative isolate (designated strain AI35) was performed by using the Illumina HiSeq2000 platform

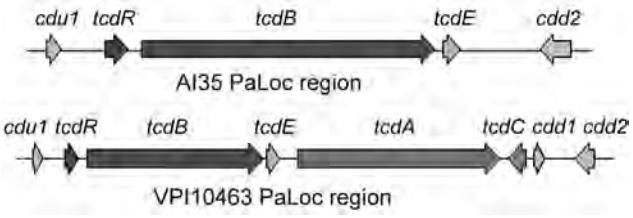

Figure 1. Structure of pathogenicity locus (PaLoc) and flanking regions in *Clostridium difficile* strains AI35 and VPI10463. Boxes indicate open reading frames; arrows indicate direction of transcription. Encoded genes are indicated above the arrows. Figure not drawn to scale.

(Australian Genome Research Facility, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia). Paired-end reads of 31,085,914 bp were assembled into 117 contigs by using the Velvet software suite (*10*).

Toxin B quantitation was performed by using a Vero cell cytotoxicity assay (*11*). Vero cells were exposed to serial 2-fold dilutions of *C. difficile* cell-free culture supernatants from a ribotype 027 human strain (M7404), a ribotype 078 animal strain (JGS6133), and strain AI35. *C. difficile* strain 630, a recognized low-toxin producer, was included as a control. After the cultures were incubated overnight at 37°C in 5% CO_2, morphologic changes were examined by microscopy. The endpoint was scored as the last dilution at which 100% cytopathic effect was observed. Assays were performed in triplicate on independent culture supernatants.

In vivo virulence was assessed by using a mouse model of CDI (*12*). Specifically, 6–8-week-old male C57/B6 mice (5 mice per strain) were force-fed 1×10^7 spores of the same *C. difficile* strains used in the Vero cell assays. Mice were housed in separate cages to avoid cross-contamination and monitored daily for weight loss and signs of disease.

Of the 185 piglets tested, *C. difficile* was isolated from 114 (62%): 70 (53%) of 131 piglets from the herds with severe scouring, 33 (77%) of 43 piglets from the herd with variable scouring, and 11 (100%) of 11 asymptomatic piglets. Isolates were clonal; all were novel PCR ribotype 237 and had a toxin profile of *tcd*A$^-$*tcd*B$^+$*cdt*A$^+$*cdt*B$^+$.

Genome sequencing of strain AI35 showed a novel pathogenicity locus (PaLoc) structure (Figure 1). A large deletion had removed the *tcdA* and *tcdC* genes and a portion of the adjacent *cdd1* gene located outside the PaLoc. Strain AI35 also encoded a variant TcdE. The AI35 binary toxin locus was complete and contained an intact copy of *cdtR*, unlike ribotype 078 isolates, which encode a *cdtR* with a premature stop codon. Strain AI35 may therefore be a more proficient binary toxin producer than ribotype 078 strains. Despite these variations, multilocus sequence typing showed that strain AI35 belongs to the same clade (clade 5) and sequence type (11) as ribotype 078 strains [strain AI15 in the report by Stabler et al. (*13*) is the same ribotype as AI35].

In vitro testing showed AI35 produced »25-fold less toxin B than did the ribotype 027 ($p = 0.0354$ by *t* test) and ribotype 078 ($p = 0.0074$ by *t* test) isolates, but AI35 showed similar toxin levels to the low toxin producing strain 630. AI35-mediated cytopathic effect on Vero cells was similar to that reported for the lethal toxin of *C. sordellii* and *C. difficile* strain 8864, a toxin A$^-$B$^+$ human strain with mutations affecting its glucosylation substrate specificity (*14*). Strain M7404 (ribotype 027) was significantly more virulent than strain AI35 ($p = 0.0001$ by log-rank [Mantel Cox] test) and strain JGS6133 ($p = 0.0002$ by log-rank [Mantel Cox] test). All mice infected with strain M7404 died (Figure 2, panel A), but mice infected with strains AI35 and JGS6133 survived (Figure 2, panel A). Still, despite low toxin production, AI35 caused significantly greater weight loss in mice

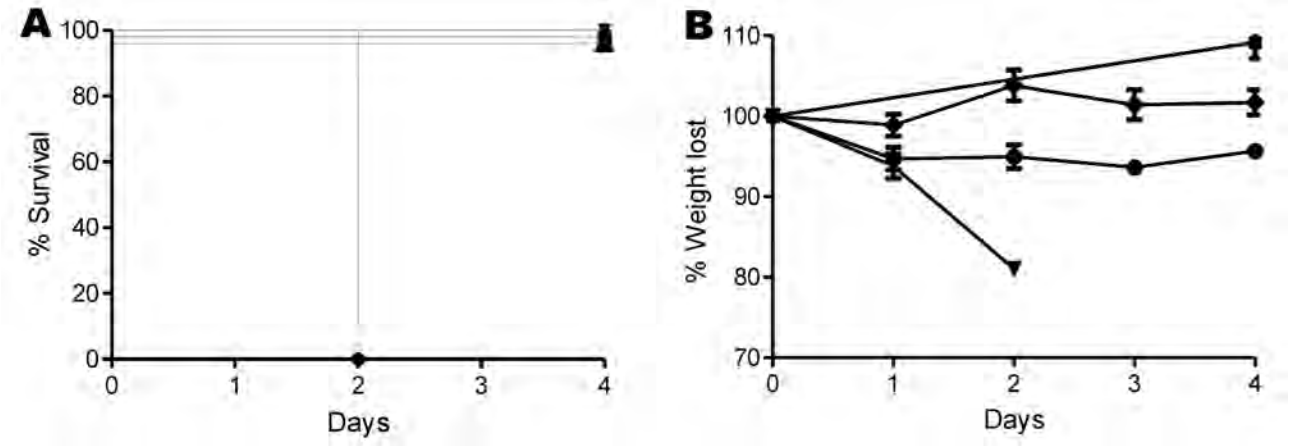

Figure 2. A) Survival and B) percentage of weight lost in mice over 4 days after infection with *Clostridium difficile*. Male C57/B6 mice were infected with *C. difficile* spores for strains M7404 (triangles), JGS6133 (078) (diamonds), or A135 (circles); phosphate-buffered saline (squares) was used as control. Error bars in panel B indicate SEM.

than did the ribotype 078 strain JGS6133 (p = 0.0011 by analysis of variance) (Figure 2, panel B). This difference may be the result of production of a variant toxin; similar toxins were 8-fold more toxic to mice than was toxin B produced by strain VPI10463 (*15*).

Conclusions

Our results show that a toxigenic *C. difficile* strain circulating in piglets in Australia is of a different ribotype, 237, than that commonly found in other parts of the world. The strain we found contained a unique PaLoc and produced more weight loss in mice than did the more common ribotype 078 animal strain. Identifying this strain is the first step in detecting and responding to this emerging disease in piglets in Australia. Future studies in swine will focus on nationwide prevalence, laboratory detection, and epidemiologic investigation to understand the transmission cycle in pigs and any relationship between animal and human disease.

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Novel Lyssavirus in Bat, Spain

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A new tentative lyssavirus, Lleida bat lyssavirus, was found in a bent-winged bat (*Miniopterus schreibersii*) in Spain. It does not belong to phylogroups I or II, and it seems to be more closely related to the West Causasian bat virus, and especially to the Ikoma lyssavirus.

Bats have been considered natural hosts of a wide diversity of viruses, including human pathogens such as lyssaviruses, severe acute respiratory syndrome coronavirus, henipavirus, and filoviruses (*1*). Within the genus *Lyssavirus*, 12 species have been described: *Rabies virus* (RABV), *Lagos bat virus* (LBV), *Mokola virus* (MOKV), *Duvenhage virus* (DUVV), *European bat lyssavirus* types 1 and 2 (EBLV-1 and -2), *Australian bat lyssavirus* (ABLV), *Aravan virus* (ARAV), *Khujand virus* (KHUV), *Irkut virus* (IRKV), *West Causasian bat virus* (WCBV), and *Shimoni bat virus* (SHIBV). Two more recently described viruses have not yet been classified: Bokeloh bat lyssavirus (BBLV) (*2*) and Ikoma lyssavirus (IKOV) (*3*).

Bats are the natural reservoirs for most lyssaviruses, and to our knowledge, only MOKV and IKOV have never been detected in bats. RABV is the only virus known to establish epidemiologic cycles in bats and carnivores, and it is responsible for most human infections, mainly transmitted by dogs. The genus *Lyssavirus* comprises at least 2 phylogroups: phylogroup I (RABV, DUVV, EBLV1–2, ABLV, ARAV, IRKV, BBLV, KHUV) and phylogroup II (LBV, MOKV, and SHIBV). Phylogroup III consists of WCBV (*4*). According to a recent phylogenetic reconstruction that included the novel IKOV and was based on a fragment of 405 nt from the nucleoprotein gene, IKOV has proven to be highly divergent (*3*) and probably also forms part of phylogroup III.

During 1977–2011 in Europe, 988 cases of bat rabies were reported to the Rabies Bulletin Europe. Bats of the species *Eptesicus serotinus* and *E. isabellinus*, which account for >95% of cases, are considered the major natural reservoirs of EBLV-1. Several bat species within the genus *Myotis* are reservoirs for EBLV-2, BBLV, and the central Asian lyssaviruses ARAV and KHUV (*5*). WCBV has been isolated in the common bent-winged bat *Miniopterus schreibersii* (*6*). Other bat species might act as eventual hosts, although in Spain, bat rabies has been declared only in *E. isabellinus* bats (*7*). The possibility of a wider host range has been suggested by some surveys on natural bat colonies of other bat species describing neutralizing antibodies and genomic fragments related to EBLV-1 (*8*).

The Study

In July 2011, a bat was found in the City of Lleida and taken to the Wildlife Care Center of Vallcalent (Lleida, Catalonia). The bat arrived lethargic and dehydrated, died soon after admission, and its carcass was frozen at -20°C. On March 12, 2012, as part of the rabies surveillance program in Spain, the bat carcass was received by the National Center of Microbiology, where rabies testing was conducted by 2 generic reverse transcription PCR (RT-PCR) methods for lyssavirus detection (*9*,*10*) and 2 commercial rabies antiserum assays (Bio-Rad Laboratories, Marnes La Coquette, France; and Fujirebio, Inc., Tokyo, Japan) for antigen detection by fluorescent antibody testing.

Brain smears were positive for lyssavirus by RT-PCR and fluorescent antibody testing, and an oropharyngeal swab sample was positive by RT-PCR. Further attempts to isolate the virus by tissue cultures were unsuccessful after 2 blind passages in BHK-21 and murine neuroblastoma cells. The negative results could be explained by the fact that the sample had been stored at –20°C for 8 months and had been frozen and thawed twice before cell culture testing; however, the possibility of the cell lines not being permissive for the virus cannot be excluded.

The bat was morphologically identified as a bent-winged bat (*M. schreibersii*) and genetically identified by cytochrome b sequencing (*11*). The genomic sequence of the corresponding fragment of the diagnostic RT-PCR on the conserved region of the nucleoprotein gene, determined by BLAST (http://blast.ncbi.nlm.nih.gov/), showed no substantial sequence similarity to previously known lyssaviruses.

To determine the identity of the lyssavirus, we sequenced a larger fragment (565 bp), including the variable codifying region of the nucleoprotein gene (GenBank accession number submitted). We reconstructed an overall

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phylogeny of lyssaviruses by using a Bayesian Inference with the first 405 nt of the N-gene and MrBayes version 3.1.2 (http://mrbayes.csit.fsu.edu/). Two simultaneous runs of 10^6 generations were conducted, each with 4 Markov chains, and trees were sampled every 100 generations. The best-fit nucleotide model, GTR + I + G, available in MrBayes,was selected according to the corrected Akaike information criterion. The phylogenetic reconstruction was based on a dataset representative of all known lyssaviruses, including the recently described IKOV. The topology obtained showed that this sequence is more closely related to IKOV and WCBV than to the lyssaviruses in phylogroups I and II (Figure). These results suggest that this sequence tentatively belongs to a new *Lyssavirus* species named after the location of collection, Lleida bat lyssavirus (LLEBV).

The highest nucleotide identity was with IKOV (71.6%), followed by SHIBV (68.6%), IRKV (68.1%), KHUV (67.6%), EBLV-2 (67%–68.2%), ARAV (67.3%), WCBV (67.4%), ABLV (66.6%–67.7%), BBLV (66.1%), LBV (65.7%–68.6%), MOKV (65.7%–67.2%), DUVV (65.5%–65.8%), RABV (64.7–66.4%), and EBLV-1 (63.7%–64%). The lowest nucleotide identity was with the only lyssavirus found in bats of the Iberian Peninsula, EBLV-1. The nucleotide identity among the previously known lyssaviruses was 63.5%–80.0% in this particular fragment, and the lowest identities among strains belonging to the same lyssavirus were 80.4% for ABLV and 79.9% for LBV (the most distant LBV strain has been suggested to be a different lyssavirus) (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1071-Techapp1.pdf).

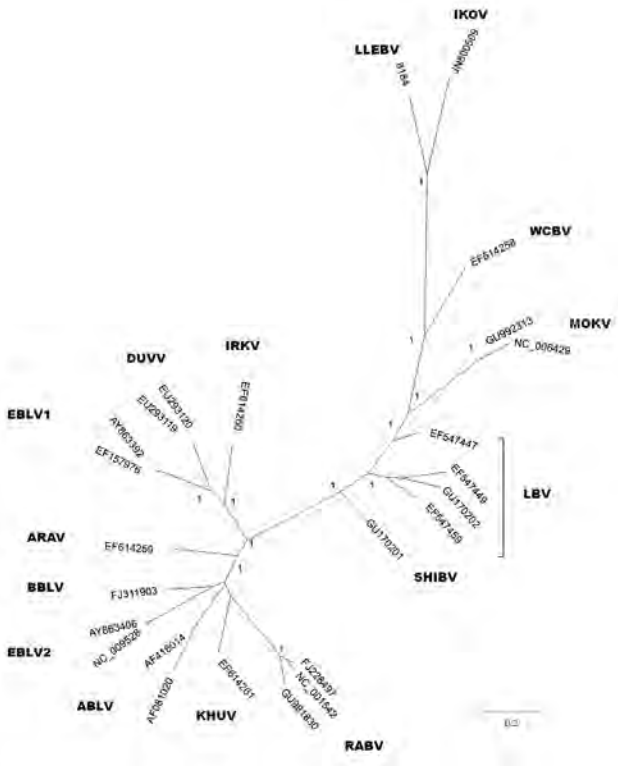

Figure. Phylogenetic reconstruction based on the first 405 nt of the nucleoprotein gene, including all representative lyssaviruses. The tree was obtained by Bayesian inference, and the first 25% of trees were excluded from the analysis as burn-in. Node numbers indicate posterior probabilities. ARAV, Aravan virus; ABLV, Australian bat lyssavirus; BBLV, Bokeloh bat lyssavirus; DUVV, Duvenhage virus; EBLV-1 and EBLV-2, European bat lyssavirus types 1 and 2; IRKV, Irkut virus; KHUV, Khujand virus; LBV, Lagos bat virus (lineages A, B, C, and D); MOKV, Mokola virus; RABV, rabies virus; SHIBV, Shimoni bat virus; WCBV, West Caucasian bat virus; IKOV, Ikoma lyssa virus; LLEBV, Lleida bat lyssavirus (proposed). Scale bar indicates expected number of substitutions per site.

Conclusions

The lyssavirus-specific antigen reactivity and association with a genomic sequence found in a bent-winged bat in northeastern Spain could be derived from the tentative new virus LLEBV. According to our phylogenetic reconstruction, the virus does not seem to belong to phylogroup I, which comprises most bat lyssaviruses, or to the African phylogroup II. The evolutionary relationships between the LLEBV sequence with WCBV and IKOV sequences need to be clarified before it can be determined whether they form >1 different phylogroups.

Of note, the new LLEBV was detected in *M. schreibersii* bats, as was WCBV, the other European lyssavirus outside phylogroup I. The genus *Miniopterus* has traditionally been considered to belong to the family Vespertilionidae as do other bat genera linked to lyssaviruses in Eurasia (*Eptesicus, Myotis*, and *Murina*). However, recent molecular analyses have confirmed that the genus *Miniopterus* belongs to the family Miniopteridae (*12*). *M. schreibersii* bats are migratory, widely distributed across southern Europe and Eurasia. Large numbers (thousands) of these bats overwinter in caves and move in the spring to different and sometimes distant summer roosts for reproduction (*13*). These ecologic features make it relatively easy for an infectious agent to quickly spread out within and among the populations.

Consequently, it is difficult to imagine that WCBV or LLEBV are locally restricted; both could be located far from where they were found. Neutralizing antibodies against WCBV have been found in bats in Africa (*14*). The cumulative description of new bat lyssaviruses in recent years shows the convenience of always using generic amplification primers for rabies diagnosis based on RT-PCR to complement antigen detection.

No human exposure to the new virus has been reported. However, because of the divergence exhibited by LLEBV and IKOV, and the growing evidence of inadequate protection/cross-neutralization against viruses outside phylogroup I, the effectiveness of current rabies vaccines for these viruses remains a concern (*15*).

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Borrelia recurrentis in Head Lice, Ethiopia

Amina Boutellis, Oleg Mediannikov, Kassahun Desalegn Bilcha, Jemal Ali, Dayana Campelo, Stephen C. Barker, and Didier Raoult

Since the 1800s, the only known vector of *Borrelia recurrentis* has been the body louse. In 2011, we found *B. recurrentis* DNA in 23% of head lice from patients with louse-borne relapsing fever in Ethiopia. Whether head lice can transmit these bacteria from one person to another remains to be determined.

Humans are the sole hosts of the pubic louse (*Pthirus pubis*), the body louse (*Pediculus humanus humanus*), and the head louse (*Pediculus humanus capitis*) (*1*). The body louse can transmit the following life-threatening forms of bacteria to humans: *Rickettsia prowazekii*, which causes epidemic typhus; *Bartonella quintana*, which causes trench fever; and *Borrelia recurrentis*, which causes louse-borne relapsing fever (*2*). Recently, DNA from *B. quintana* has been found in head lice from Nepal (*3*), the United States (*4*), France (*5*), Senegal (*6*), and the highlands of Ethiopia (Gibarku and Tikemit Eshet) (*7*). Louse-borne relapsing fever is among the top 10 causes of hospital admissions in Ethiopia and is associated with substantial illness and death (*8*). Infection of head lice with *B. recurrentis* or *R. prowazekii* has not been reported. Our aim was to assess the presence of *Borrelia, Rickettsia,* and *Bartonella* spp. in head lice and body lice from persons in the highlands of Ethiopia, where an outbreak of relapsing fever is ongoing.

The Study

In August 2011, we enrolled 24 patients (23 male, 1 female) at Bahir Dar Hospital, Ethiopia, whose blood smears were positive for *Borrelia* spp. by microscopy with Giemsa staining. After receiving permission from the patients, we collected samples of head and body lice by searching their heads and clothing. Lice were randomly selected, preserved in 100% ethanol, and taken to the reference center at Marseille Université, Marseille, France. Each louse was rinsed 2× in sterile water. Genomic DNA was extracted by using the QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany), as recommended by the manufacturer, and stored at -20°C.

Quantitative real-time PCR (qPCR) was performed by using primers and probes that targeted a portion of the *Bartonella* 16S–23S intergenic transcribed spacer region and a specific *B. quintana* gene, *yopP*, which encodes for a putative intracellular effector (*5*). For a specific *R. prowazekii* gene, we used previously described primers and probes (*9*), and for *Borrelia* spp., we used previously described primers and probes selective for the 16S rRNA gene (*10*). To confirm the positive qPCR results, we performed a standard PCR that used primers for an intergenic spacer region between the 16S rRNA gene and a gene encoding a hypothetical protein of *B. recurrentis*. The primers used for this experiment were B.rec F: 5′-TTCGCCACTGAATGTATTGC-3′ and B.rec R: 5′-TGCCAATGTTCTTGTTGGTC-3′ (*11*). Uninfected body lice (Orlando strain) were used as negative controls for each test.

Among the 24 patients, 11 had head and body lice, 11 had body lice only, and 2 had head lice only. Classification of lice was based on phenotype (head lice, black; body lice, gray), ecotype (found on head and hair or in clothing), and cytochrome b genotype (clade) (data not shown) (*1*). Some head lice were found on the patients' clothing, specifically on collars and hats.

Totals of 35 head lice and 62 body lice were tested individually. *Borrelia* spp. DNA was found in 8 (23%) head lice from 5 patients and in 25 (40%) body lice from 15 patients (Table). *B. quintana* DNA was found in 1 (3%) head louse and in 7 (11%) body lice. *Borrelia* spp. and *B. quintana* were found in 5 (8%) body lice from 3 patients (Table). DNA from *R. prowazekii* was not found in any of the 97 lice.

For the 11 patients who were infested with head and body lice (29 of each type), prevalence of *Borrelia* spp. DNA was significantly higher among the body lice than among the head lice (16 [55%] of 29 vs. 6 [21%] of 29; p = 0.006) (Table). *B. quintana* DNA was found in 1 (3%) head louse and in 4 (14%) body lice, but the difference was not significant. For the 11 patients infested with body lice only, 9 (27%) of 33 body lice were positive for *Borrelia* spp. and 3 (9%) were positive for *B. quintana*. For the 2 patients infested with head lice alone, 2 (33%) of 6 head lice were positive for *Borrelia* DNA. The *Borrelia* DNA that was in 4 head lice and 5 body lice (collected from 9 patients) was then used to identify the species of *Borrelia* that infected these patients. For this identification, we performed a pairwise comparison of the intergenic spacer sequence from the putative *Borrelia* species in these

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Table. Results of quantitative real-time PCR analysis of head lice and body lice from 24 patients, Bahir Dar, Ethiopia, 2011*

Patient no.	Patient sex	No. head lice	No. body lice	*Bartonella quintana*		*Borrelia recurrentis*	
				In head lice	In body lice	In head lice	In body lice
Head and body lice							
1607007	M	3	2	0	0	0	0
1607008	M	2	1	0	0	1 (C_t 34)	1 (C_t 25)
1607009	M	1	3	0	0	0	0
16070010	M	3	3	0	1 (C_t 35)	0	1 (31)
1807002	M	2	3	0	0	2 (C_t 34)	3 (C_t 35)
1907002	M	3	3	0	0	1 (C_t 33)	3 (C_t 30)
2007001	M	3	2	1 (C_t 32)	0	0	1 (C_t 35)
2007002	M	3	3	0	0	0	2 (C_t 34)
2107001	M	3	3	0	0	0	2 (Ct 27)
2107002	M	3	3	0	0	0	1 (C_t 34)
2307002	M	3	3	0	3 (C_t 27)	2 (C_t 34)	2 (C_t 31)
Total	11	29	29	1 (3.44%)	4 (13.8%)	6 (20.70%)	16 (55.17%)
Body lice only							
1607001	M	0	3	0	0	0	1 (C_t 29)
1607002	M	0	3	0	0	0	1 (C_t 35)
1607003	M	0	3	0	0	0	1 (C_t 21)
1607004	M	0	3	0	2 (C_t 36)	0	2 (C_t 27)
1607005	M	0	3	0	0	0	0
1607006	M	0	3	0	0	0	0
1707001	M	0	3	0	0	0	1 (C_t 35)
1907003	M	0	3	0	0	0	3 (C_t 31)
2407001	F	0	3	0	0	0	0
2407003	M	0	3	0	0	0	0
2407004	M	0	3	0	1 (C_t 36)	0	0
Total	11	0	33	0	3 (9.0%)	0	9 (27.27%)
Head lice only							
1807001	M	3	0	0	0	2 (C_t 31)	0
1907001	M	3	0	0	0	0	0
Total	2	6	0	0	0	2 (33.33%)	0

*C_t, cycle threshold.

patients with *B. recurrentis*, *B. duttonii*, and *B. crocidurae* sequences from GenBank; results showed 100%, 97%, and 93% similarities, respectively (GenBank accession nos. JX126797–JX126805).

We detected *B. recurrentis* DNA in head and body lice by using qPCR and confirmed these results by sequencing the amplicons. Among patients infested with head and body lice, the numbers of body lice infected with *B. recurrentis* were substantially higher than the numbers of head lice infected with *B. recurrentis*; however, *B. recurrentis* DNA was also found in lice from patients infested with head lice alone.

Conclusions

Human head and body lice are generally thought to colonize their hosts in different ways. However, head and body lice are often both found on heavily infested persons and might migrate from head to body and vice versa (*12*). Head lice in Ethiopia are black and belong to the cytochrome b clade (genotype) C, whereas body lice are gray and belong to clade A (*7*). We also found that head lice from heavily infested patients were in physical proximity (i.e., on the collars of clothing) with body lice.

Body lice are the principal vectors of *B. recurrentis* (*2*). However, head lice can also be present in large numbers on persons with body lice because the conditions that lead to prevalent and prolonged infestations with body lice–such as poverty, inability to change clothes, and crowding–also favor head lice (*5*). We hypothesize that in patients who are simultaneously infested with both types of lice, the head lice might be contaminated with blood containing *Borrelia* spp.

The transmission of relapsing fever to humans occurs by the rupturing of a louse and subsequent inoculation by scratching because *Borrelia* spp. are found in the hemolymph of the insect. Recently, however, viable *B. recurrentis* was found in lice excrement, which implies that the organism might return to the gut from the hemolymph (*13*).

In conclusion, head lice from patients with louse-borne relapsing fever were infected with *B. recurrentis* and *B. quintana*. Whether head lice can transmit these pathogenic bacteria from person to person remains to be explored. To determine whether *B. recurrentis* and *B. quintana* occur in head lice, epidemiologic studies of head lice collected from more patients with louse-borne relapsing fever and trench fever should be conducted.

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Tuberculosis Exposure among Evacuees at a Shelter after Earthquake, Japan, 2011

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Tuberculosis was diagnosed in a person who had stayed in a shelter after the 2011 Great East Japan Earthquake. A contact investigation showed that the prevalence of latent tuberculosis infection among other evacuees at the shelter was 20%. Our report underscores the importance of tuberculosis prevention and control after natural disasters.

In the aftermath of the March 11, 2011, Great East Japan Earthquake and subsequent tsunami, many survivors were forced to live in shelters under harsh and unsanitary conditions. The occurrence and outbreaks of infectious diseases at shelters after the earthquake were of concern because conducting standard precautions was difficult and access to health care was poor. Influenza outbreaks and an increase in pneumonia cases in shelters were reported after the earthquake (*1*,*2*), and infection control activities were required to support shelters in efforts to minimize infectious diseases (*3*). We report a case of active pulmonary tuberculosis (TB) in a person who stayed at a shelter after the 2011 Great East Japan Earthquake and the results of an investigation of the prevalence of latent tuberculosis infection (LTBI) among evacuees and others who were exposed to this patient.

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The Study

The index case-patient was an 87-year-old woman with congestive heart failure who was referred from a disaster medical assistance team and admitted to our hospital on April 6. She reported leg edema starting at the end of February and a cough beginning in early March. On March 11, the day of the earthquake and resulting tsunami, she spent a night in a shrine near her home, which had been completely destroyed. She had also lost all of her daily medications. She stayed at a disaster shelter with family members and other evacuees on March 12–15 (3 days) and then moved to her daughter's home. She reported that she experienced fever, headache, general malaise, appetite loss, "terrible" cough, sputum, and dyspnea on April 4; on April 6, she visited the disaster medical assistance team.

Chest radiograph and computed tomography scan were performed, and results revealed extensive infiltrative shadows with air bronchograms in the right lung; results were normal for the left lung. Pneumonia was diagnosed, and an empirical therapy of intravenous antimicrobial drugs was initiated, pending further evaluation for pulmonary tuberculosis. Sputum smear testing showed acid-fast bacilli (AFB) graded Gaffky 2 (grade 1+ on the World Health Organization scale) (*4*). *Mycobacterium tuberculosis* complex infection was identified by PCR. The patient was placed in a negative-pressure single room and treated with a 3-drug regimen of isoniazid, rifampin, and ethambutol. Sputum culture yielded *M. tuberculosis* susceptible to the drugs administered. The patient improved and was discharged on June 17 after sputum smears were repeatedly negative for AFB. She continued to receive directly observed treatment at our outpatient clinic and completed 9 months of treatment.

The shelter at which the patient stayed after the earthquake was small (60 m^2), and ≈50 evacuees stayed there at the time she was there. Ventilation was poor because the weather was cold and windows were not opened. Mask supply was insufficient, and most persons did not wear masks or did not wear them properly. Obtaining information on persons who had contact with the index case-patient was difficult because many evacuees were exhausted from stress or had moved to secondary shelters or relatives' home by the time we visited the shelter. In cooperation with a manager of the shelter and local government, a contact investigation for the index case-patient patient was conducted during June–August 2011 (2–4 months after the last possible exposure) to identify LTBI; a total of 62 contact persons were found. Three contacts

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(an infant and children <7 years of age) had a tuberculin skin test (TST), and the remaining 57 contacts (persons >7 years of age) underwent whole-blood interferon-γ release assay (IGRA) testing by using QuantiFERON-TB Gold In-Tube (Cellestis, Chadstone, Victoria, Australia), as described (*5*).

Two of 3 contacts tested by TST were positive for tuberculin purified protein derivative (PPD); 9 of 57 contacts tested by IGRA were positive (Table). A 2-month-old infant, a family member of the index case-patient, had been initially tested in May and was PPD negative but received isoniazid therapy for LTBI because the infant had close and frequent contact with the index case-patient. Two months after the 6 months of treatment was completed (8 months after treatment was initiated), results of a repeat TST on this infant were positive.

For PPD- or IGRA-positive contacts, medical examinations and chest radiographs were performed, but no results characteristic of pulmonary TB were found. After a physician explained risks and benefits of prophylaxis to the contacts (or parents), 8 contacts (7 evacuees and 1 family member) received prophylactic treatment for LTBI; the other contacts received follow-up chest radiography. One year after the earthquake, no active TB cases had been observed among the contacts.

Conclusions

We detected LTBI among evacuees who were exposed to a patient with active TB at a shelter after the 2011 Great East Japan Earthquake. Refugees and populations displaced after natural disasters are particularly vulnerable to TB in developing countries because crowded living conditions and poor nutritional status can facilitate the development and the transmission of TB (*6*,*7*). However, as seen in this report and others (*8*), displaced populations in industrialized countries may also be vulnerable to communicable diseases after natural disasters. Persons in shelters who have TB or suspected TB should be transferred to a medical facility as soon as the illness is detected because isolation and respiratory protection for airborne diseases such as TB is very difficult to implement in shelters (*9*).

In our case, some physicians and nurses who saw the index patient did not consider the diagnosis of TB or could not perform AFB testing, which may have delayed time to TB diagnosis and resulted in TB spread in the shelter. Health care personnel working in disaster relief must suspect and rapidly diagnose TB and then conduct contact investigations in collaboration with local public health departments. Signs and symptoms and clinical characteristics of TB in the elderly may be atypical (*10*), so continuous medical education and training are needed to maintain the competence of health care personnel to prevent, diagnose, and treat TB in the elderly.

Table. Sample results from investigation of TB exposure among evacuees at shelter and relatives who had contact with index case-patient after earthquake, Japan, 2011*

Contact type	Age, y/sex	TB screening†		Intervention
		Type	Result	
Evacuee‡	85/M	NA	NA	Follow-up
Evacuee‡	57/F	IGRA	Negative	Complete
Evacuee‡	32/M	IGRA	Negative	Complete
Family	57/M	IGRA	Negative	Complete
Family	55/F	IGRA	Negative	Complete
Family	30/M	IGRA	Negative	Complete
Family	32/F	IGRA	Negative	Complete
Family	11/F	IGRA	Negative	Complete
Family	4/F	TST	Positive	Follow-up
Family	<1/F	TST	Negative	LTBI treatment
Evacuee	64/M	IGRA	Negative	Complete
Evacuee	61/F	IGRA	Negative	Complete
Evacuee	29/M	IGRA	Negative	Complete
Evacuee	56/F	IGRA	Negative	Complete
Evacuee	86/F	IGRA	Negative	Complete
Evacuee	48/M	IGRA	Negative	Complete
Evacuee	54/F	IGRA	Borderline	Follow-up
Evacuee	63/F	IGRA	Negative	Complete
Evacuee	73/M	IGRA	Borderline	Follow-up
Evacuee	59/F	IGRA	Negative	Complete
Evacuee	60/M	IGRA	Negative	Complete
Evacuee	56/M	IGRA	Negative	Complete
Evacuee	63/M	IGRA	Borderline	Follow-up
Evacuee	60/F	IGRA	Borderline	Follow-up
Evacuee	74/M	IGRA	Positive	Follow-up
Evacuee	67/F	IGRA	Negative	Complete
Evacuee	73/M	IGRA	Borderline	Follow-up
Evacuee	40/M	IGRA	Borderline	Follow-up
Evacuee	38/F	IGRA	Negative	Complete
Patient	81/F	IGRA	Negative	Complete
Evacuee	76/M	IGRA	Positive	Follow-up
Evacuee	70/F	IGRA	Negative	Complete
Evacuee	65/M	IGRA	Negative	Complete
Evacuee	62/F	IGRA	Positive	LTBI treatment
Evacuee	43/M	IGRA	Positive	LTBI treatment
Evacuee	75/F	IGRA	Negative	Complete
Family	28/M	IGRA	Negative	Complete
Evacuee	73/M	IGRA	Negative	Complete
Evacuee	62/F	IGRA	Positive	Follow-up
Evacuee	91/F	IGRA	Positive	LTBI treatment
Evacuee	91/F	IGRA	Positive	LTBI treatment
Evacuee	78/M	IGRA	Positive	LTBI treatment
Evacuee	79/F	IGRA	Negative	Complete
Evacuee	49/M	IGRA	Positive	LTBI treatment
Evacuee	38/M	IGRA	Negative	Complete
Evacuee	70/M	IGRA	Negative	Complete
Evacuee	66/F	IGRA	Borderline	LTBI treatment
Evacuee	42/F	IGRA	Negative	Complete
Evacuee	13/F	IGRA	Negative	Complete
Evacuee	7/M	TST	Positive	Follow-up
Evacuee	74/F	IGRA	Negative	Complete
Patient	96/F	IGRA	Negative	Complete

*A complete list of contact persons is available in the expanded Table online (wwwnc.cdc.gov/EID/article/19/5/12-1137-T1.htm). Contact persons were 50 evacuees (3 family members and 47 others) at the shelter, 8 family members at the index case-patient's daughter's home, 2 health care personnel (rescue worker and doctor) who rode with the patient in an ambulance, and 2 patients admitted to the same room as the index case-patient in the hospital. TB, tuberculosis; NA, not available; IGRA, interferon-γ release assay; TST, tuberculin skin test; LTBI, latent tuberculosis infection; border, borderline.
†TST or IGRA results were not available for 2 contacts; 1 refused to receive IGRA, and 1 did not undergo testing because of advanced age. IGRA results were evaluated according to the Japanese guideline for using the QuantiFERON-TB Gold In-Tube (*4*).
‡Family member of index case-patient.

In this investigation, TST or IGRA results were positive (indicating LTBI) in 11 (18.3%) of 60 contacts; 10 (20%) of 50 evacuees at the shelter had positive results. A previous study in Japan found that the IGRA-positive rate was 7.1% for those 40–69 years of age in the general population (*11*). Japan is considered a middle-burden TB country and has a bacille Calmette-Guérin vaccination program; for these reasons, IGRA is more useful than TST in LTBI screening and contact tracing.

Adherence rates for LTBI treatment were low (19%) at a TB clinic in New Orleans after Hurricane Katrina (*8*). This finding indicates a challenging environment for TB control activities after a natural disaster and suggests an increased risk for transmission because of migration and overcrowding. Disrupted health care services, poor access to TB control programs, and difficulty in patient management may lead to poor treatment adherence, which could result in the emergence of drug-resistant TB strains. However, all (8/8, 100%) patients with LTBI who initiated treatment in our investigation adhered to and completed the regimen. Our success in postdisaster TB control measures at this shelter can be attributed, in part, to the efforts of public health nurses in providing education and directly observed treatment despite limited resources and poor health care access in the affected area. Medical institutions and public health departments should work to cooperatively and collaboratively assist shelter in implementing TB care and control activities for evacuees after natural disasters.

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Genetic Analysis of Primaquine Tolerance in a Patient with Relapsing Vivax Malaria

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Patients with *Plasmodium vivax* malaria are treated with primaquine to prevent relapse infections. We report primaquine failure in a patient with 3 relapses without any possibility of re-infection. Using whole genome sequencing of the relapsing parasite isolates, we identified single nucleotide variants as candidate molecular markers of resistance.

Of the 5 species of *Plasmodium* that cause human malaria, *P. vivax* has the broadest geographic distribution with 2.85 billion persons at risk throughout the world (*1*). Scientists are becoming increasingly aware of the potential severity of *P. vivax* infections and their effects on public health (*2*). A major challenge is the treatment of the dormant stages, hypnozoites, in the liver. Activation of hypnozoites from this reservoir causes subsequent blood-stage infections, or relapses, weeks to years after the primary infection.

Primaquine (PQ) remains the only approved agent to eliminate hypnozoites. Treatment failure, defined by the occurrence of relapses despite PQ therapy, is often ascribed to inadequate dosing, poor adherence, or reinfection (*3*). However, several cases of PQ tolerance without these confounding factors are reported (*4*,*5*). The mechanism underlying PQ tolerance is not understood, although host and parasite genetic factors are implicated. We describe the genetic analysis of parasite and host markers in a patient with 3 *P. vivax* malaria relapses in a malaria-nonendemic setting where reinfection was not possible.

The Case

The patient is a 38-year-old man from northeast Africa. In December 2008, he experienced a febrile illness in Sudan that was diagnosed as vivax malaria. He was treated with chloroquine (CQ) but did not receive PQ. The patient recovered and moved to Canada in mid-January 2009. One month after his primary infection, he sought treatment at a hospital in Canada with fever, chills, and malaise. *P.vivax* malaria was diagnosed by microscopy and real-time polymerase chain reaction. He was treated with CQ (600 mg base immediately, 300 mg base at 6, 24, and 48 h), followed by 14 d of PQ (30 mg by mouth daily). His estimated weight was 60 kg. The patient's symptoms resolved, and smears were negative for *Plasmodium* on day 16. The patient experienced a second episode of symptomatic *P. vivax* malaria 3 months later. He was treated with CQ as before, followed by 28 days of PQ (30 mg by mouth daily). Smears were negative 2 days later. Nearly 30 months later, the patient had a third episode of *P. vivax* malaria. He had not traveled outside North America since his arrival in Canada. He was treated with CQ for 3 d, then PQ for 14 d (30 mg by mouth daily). Smears on days 2 and 9 after CQ treatment were negative. The importance of adherence was emphasized at each clinic visit, and the patient affirms that he took the full course of PQ treatment at the same time every day.

To identify mutations in parasite genes that are potentially associated with primaquine tolerance, we performed whole genome sequencing on *P. vivax* DNA obtained from patient samples at each relapse (EAC01–03). In total, 55,517 high-confidence single nucleotide variants (SNVs) were genotyped (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1852-Techapp1.pdf). The 3 parasite isolates were genetically related, but not identical, and they have been proposed to be meiotic siblings (A.T. Bright et al., unpub. data).

In addition, the 3 strains contained SNVs in genes homologous to known *P. falciparum* drug-resistance genes, including *pvdhps*, *pvmdr*, and *pvmrp* (*6*–*8*). Variants compared to the *P. vivax* reference strain SalI, presumed to be primaquine sensitive, were found at 27 of 39 sites within 5 known and putative drug resistance genes (Table). All 3 isolates possessed a double mutant antifolate-resistant genotype in *pvdhfr* (*6*). The SNVs within the putative drug-resistance genes in each of the patient's 3 samples were identical except at amino acid positions 976 and 1393 of the *pvmdr1* gene. The parasite genomes were also

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Table. Genetic polymorphisms in drug-resistance genes from relapsing isolates of *Plasmodium vivax* *

Gene	Chromosome	Polymorphism	Amino acid	Reference	Brazil	EAC01	EAC02	EAC03
pvcrt	1	T330262C	5′ UTR	T	C	C	ND	C
PVX_087980		T330482C	5′ UTR	T	T	T	T	T
		G330484T	5′ UTR	G	G	G	G	G
		C330495A	5′ UTR	C	C	C	C	C
		T331151C	Intron	T	C	C	C	C
		T332453C	Intron	T	C	C	ND	C
		A332874C	Intron	A	C	C	ND	C
		G333391A	Intron	G	G	A	A	A
		A333518G	Intron	A	A	G	G	G
		T333544C	Intron	T	T	C	C	C
pvmrp	2	G153936A	F1629	G	G	ND	A	A
PVX_097025		C154067T	H1586Y	C	C	C	C	C
		G154391A	V1478I	G	A	ND	A	A
		G154567C	G1419A	G	G	G	G	G
		T154646G	Y1393D	T	G	ND	G	G
		T154979A	L1282I	T	T	T	T	T
		T155127G	I1232	T	T	ND	G	G
pvdhfr	5	C964763G	S58R	C	A	G	G	G
PVX_089950		T964796C	Y69	T	C	T	T	T
		G964939A	S117N	G	A	A	A	A
		G964970A	P127	G	G	A	A	A
		A965106C	I173L	A	C	A	A	A
pvmdr1	10	C361917G	K1393N	C	C	C	C	G
PVX_080100		T362031C	K1355	T	T	T	T	T
		A362870G	F1076L	A	A	G	G	G
		G363032A	L1022	G	G	G	G	G
		T363169A	Y976F	T	T	A	A	T
		G363223A	T958M	G	A	A	A	A
		T363374G	M908L	T	G	G	G	G
		G363563A	L845F	G	G	G	G	G
		C364004T	G698S	C	C	T	T	T
		T364509C	T529	T	C	C	C	C
		A364557T	S513R	A	A	T	T	T
		C364598T	D500N	C	T	C	C	C
pvdhps	14	G1256840A	Intron	G	G	A	A	A
PVX_123230		T1257042C	P654	T	T	C	C	C
		G1257064A	A647V	G	G	A	A	A
		G1257856C	A383G	G	C	C	C	C
		G1257859C	S382C	G	C	G	G	G
		C1258389T	M205I	C	T	T	T	T
		T1258579C	E142G	T	T	C	C	C

*UTR, untranslated region; ND, not determined

compared to the BrazilI strain of *P. vivax,* which was obtained from a patient who had multiple malaria episodes in a malaria-nonendemic country despite primaquine treatment (*9*). Comparison of the genotypes at the 5 genes demonstrated similar profiles. All strains exhibit intermediate to high levels of antifolate resistance on the basis of mutant genotypes identified in *pvdhfr* and *pvdhps*. In addition, the parasite strains obtained in this study share variant alleles with BrazilI in 2 multidrug resistance–associated transporters, *pvmdr* and *pvmrp*.

Host pharmacogenetics may also contribute to PQ failure by affecting drug metabolism. Genetic polymorphisms in the *CYP* gene family are associated with poor or intermediate metabolism of many drugs used to treat tropical infections (*10*) and several of these enzymes are specifically implicated in the metabolism of PQ (*11*) and other antimalarial drugs (*12*). We, therefore, determined whether the patient carried alleles that might also explain the failure of treatment. Based on allele frequencies in northeasernt African populations, polymorphisms within 4 of the 60 *CYP* genes were selected for genotyping: *CYP1A2*1C*, *CYP2B6*6*, *CYP3A4*1B*, and *CYP2D6*4* (online Technical Appendix). The patient was homozygous for the wild-type allele at all 4 loci.

Lastly, we examined whether the patient metabolized PQ to carboxy-primaquine (CPQ), the main PQ metabolite found in plasma. Drug levels were measured with a stereoselective bioanalytical LC-MS/MS method (W. Hanpithakpong et al., unpub. data). A plasma sample was collected on day 12 of treatment of the 3rd relapse, at 12–15 h post-dose. The total PQ and CPQ concentrations were 90 ng/mL and 1,042 ng/mL, respectively. The measured PQ concentration was similar to simulated maximum concentrations at steady-state in healthy male volunteers (96 ng/mL) and patients with vivax malaria (88 ng/mL). Concentration-time profiles for CPQ could not

be simulated because of limited published information. These data demonstrate appropriate absorption of PQ and metabolism into CPQ.

Conclusions

Although this case highlights the challenges in managing patients with *P. vivax* who relapse after high doses of PQ, it also provides a unique opportunity to clarify the mechanisms underlying PQ tolerance. The multiple relapses in this patient result from previously acquired hypnozoites that likely possessed a genetic profile rendering them tolerant to PQ. Genotyping did not identify any mutations within 4 of the *CYP* loci potentially responsible for the antiparasite effect of PQ and plasma measurements demonstrated adequate levels of PQ and CPQ. However, this study presents a limited screen of polymorphisms in the *CYP2D6* gene (*13*), and we cannot exclude the possibility that other alleles contribute to PQ tolerance.

Parasite genotype data demonstrate that the 3 isolates contain mutations in several putative drug- resistance genes. All 3 isolates are resistant to antifolates and harbor mutations in the ABC transporter genes that are implicated in resistance to numerous antimalarial drugs. Of particular interest are the mutations in the *pvmrp1* gene that encodes a putative multidrug resistance-associated protein. Studies from *P. falciparum* implicate PfMRP1 in glutathione efflux, consistent with the predicted mode of action of PQ in disrupting mitochondrial function (*14*). Furthermore, gene knockouts of *pfmrp1* have increased sensitivity to several antimalarial drugs, including PQ, which suggests this protein may play a role in transporting antimalarial agents out of the parasite (*15*).

This case study demonstrates the feasibility of using molecular tools to better understand therapeutic responses to PQ. Genetic analysis of SNVs in putative resistance genes may identify molecular markers of parasite resistance or correlate with known variations in PQ sensitivity of strains from different geographic areas. Clarification of the role of genetic factors involved in PQ efficacy cannot be readily addressed in populations in which endemic transmission occurs because relapses cannot be distinguished from reinfections. Genetic studies of relapses that occur in nontransmission settings provide a unique opportunity to answer questions about this human pathogen.

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Tick-borne Encephalitis Associated with Consumption of Raw Goat Milk, Slovenia, 2012

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Tick-borne encephalitis (TBE) developed in 3 persons in Slovenia who drank raw milk; a fourth person, who had been vaccinated against TBE, remained healthy. TBE virus RNA was detected in serum and milk of the source goat. Persons in TBE-endemic areas should be encouraged to drink only boiled/pasteurized milk and to be vaccinated.

In Europe, tick-borne encephalitis (TBE) is one of the most common flavivirus infections of the central nervous system and is endemic to several countries. Slovenia is among European countries with the highest reported TBE incidence rates (8.1–18.6 cases/100,000 population in the past decade) (*1*). TBE virus (TBEV) is mainly transmitted by tick bites but occasionally is transmitted by ingestion of unpasteurized milk/milk products from infected livestock (*2*).

Previously, large TBE outbreaks linked to a common source had been associated with consumption of dairy products (mostly goat milk); in recent years, smaller, dairy product–associated outbreaks have been reported from several TBEV-endemic countries (*3*–*6*). Despite high TBE incidence rates and low uptake of TBE vaccine among the Slovenian population (*7*), alimentary transmission of TBEV had not been reported in the country. We report a small outbreak of TBE that occurred in 2012 among persons in Slovenia who consumed raw goat milk.

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The Study

On May 8, 2012, acute symptomatic TBEV infection was diagnosed in a kidney transplant patient in Slovenia (Table, Patient 1). A possible link between the infection and consumption of raw goat milk was revealed, triggering a detailed investigation of possible sources of infection and of 3 other persons who, together with patient 1, had consumed ≈2 L of raw milk (colostrum) from the same goat on April 18 (Table). Two days after the milk was consumed, fever, fatigue, and malaise developed in 3 of the 4 person, including Patient 1, who also had headache and myalgia.

Patient 3 did not seek medical care. Patients 1 and 2 were examined in the emergency department of the local general hospital on April 20. Laboratory test results were in the reference range, with the exception of mild leukopenia in both patients and mildly elevated liver enzyme levels for Patient 1. TBE was not suspected at that time. All 3 patients recovered in <1 week. Patient 3 remained well, but a second phase of disease developed in Patients 1 and 2 approximately 14 days after the milk was consumed. The second phase was characterized by high fever, headache, nausea (and vomiting in Patient 1), tremor, and mild disturbances of concentration and consciousness. Results of cerebrospinal fluid laboratory tests for Patient 2 revealed abnormalities consistent with aseptic meningitis (reference values are in parentheses): leukocytes 29 × 10^6/L (<5 × 10^6/L), neutrophilic granulocytes 9 × 10^6/L (<5 × 10^6/L), lymphocytes 20 × 10^6/L (<5 × 10^6/L), protein concentration 0.39 g/L (0.15–0.45 g/L), glucose concentration 3.27 mmol/L (2.5–3.9 mmol/L). Patient 1, who refused lumbar puncture diagnostic testing and hospitalization, was treated as an outpatient. The course of disease in Patients 1 and 2 was moderately severe, and the outcome was favorable. A detailed epidemiologic history revealed that none of the 3 patients recalled a recent tick bite and that Patients 2 and 3 consumed raw goat milk rather often, believing it was healthful.

For all 3 patients, TBEV infection was confirmed by 1) ELISA (Enzygnost Anti-TBE/FSME Virus [IgG, IgM]; Siemens, Marburg, Germany) demonstrating specific IgM and IgG against TBEV in serum and by 2) the presence of neutralizing antibodies against TBEV. Real-time reverse transcription PCR of serum samples did not detect TBEV RNA (Table) (*8*).

The fourth person, who remained healthy, was previously vaccinated against TBE. He received his basic vaccination (3 doses) during 1995–1996, the first booster dose in 2000, the second in 2005, and the third in 2010. Serologic test results showed the absence of specific IgM and high levels of specific IgG. An antibody concentration of 912 U/mL in the first serum sample, obtained 27 days after he consumed raw goat milk, and of 672 U/mL in the second serum sample, obtained 3 weeks later, together with

Table. Epidemiologic characteristics of persons in whom tick-borne encephalitis developed after drinking raw goat milk. Slovenia, 2012*

Patient no., age, y/sex	Date(s) milk consumed	Illness phase		Date(s) serum sample obtained	Virologic testing			
		First	Second		TBEV ELISA		TBEV NT	TBEV rRT-PCR
		Onset date; clinical signs; duration	Onset date; clinical signs; duration		IgM	IgG		
1, 31/M	Apr 18	Apr 20; fever (38.0°C), chills, headache, vomiting, muscle aches, sore throat, sensitivity to light; nearly 1 wk	May 5; fever (39.8°C), headache, nausea, vomiting, photophobia, poor concentration, blurred vision, tremor; improvement after 8 d	May 8	Pos	Pos	Pos	Neg
2, 59/F	Apr 18	Apr 20; fever (≤38.6°C), chills, malaise, loose stools; 5 d	May 3; fever (38.5°C) for 6 d, headache, nausea, confusion, visual disturbances, tremor; marked improvement after 12 d	May 8	Pos	Pos	Pos	Neg
3, 32/M	Apr 17, 18, 20	Apr 20; fever (39.5°C); chills, fatigue, muscle pain; 4 d	Not ill	May 15	Pos	Pos	Pos	Neg
				Jun 6	Pos	Pos	Pos	ND
4, 28/M	Apr 18	Not ill	Not ill	May 15	Neg	Pos	Pos	Neg
				Jun 6	Neg	Pos	Pos	ND

*Except for patient 4, no patients were vaccinated against tick-borne encephalitis. For patients 1 and 2, the incubation period was 2 d; for patient 3, the incubation period was 2–3 d. Patient 1 refused hospitalization and was treated as an outpatient; patient 2 was hospitalized for 8 d; patient 3 did not seek medical care. TBEV, tick-borne encephalitis virus; NT, neutralization test; rRT-PCR, real-time reverse transcription PCR; Pos, positive; Neg, negative; ND, not done.

a high relative avidity index (85%), suggested a recent booster response.

Patient 3, the owner of a small farm with 9 sheep and 9 goats, including the goat whose milk was consumed, consented to a virologic investigation of serum, blood, and milk samples from his farm animals. By using an indirect immunofluorescent assay, we detected TBEV-specific antibodies in 5 of 9 goat serum samples (titer range 20–1,280) and in 1 of 4 goat milk samples. All samples from sheep were seronegative for TBEV. Quantitative real-time reverse transcription PCR for TBEV was performed on all serum and blood samples and on 4 goat milk samples (*9*). TBEV RNA was detected in serum (1.50×10^3 RNA copies/mL) and milk (1.88×10^5 RNA copies/mL) of the goat whose milk was consumed, confirming the source of infection. TBEV RNA was not detected in samples from the other farm animals.

Conclusions

Our investigation of illness among 3 of 4 persons who consumed TBEV-infected raw goat milk revealed that all 4 persons were infected with the virus. Febrile illness developed in 3 of the 4 persons 2–3 days after the milk was consumed; the fourth person, who had been vaccinated against TBE, remained healthy. The course of the illness was biphasic in 2 of the 3 symptomatic persons: leukopenia (a characteristic finding for the initial phase of TBE) was present during the initial phase, and the second phase was clinically indicative of meningoencephalitis. Even though 1 of these 2 patients received immunosuppressive therapy because of renal transplantation, the course of the disease was only moderately severe, and the outcome was favorable. A prospective clinical study of patients in the initial phase of TBE who were monitored for the appearance of the second, encephalitic phase of the disease, showed that an abortive form of TBE (i.e., an isolated initial phase not followed by the meningoencephalitic phase), as seen in the third patient in our study, is a rare event (*10*).

After a tick bite, the incubation period for TBE is a median of 8 days (range 4–28 days) (*2*). The incubation period can be shorter for exposure by the alimentary route (*5*) and was found to be only 2 days in the patients in our study. These findings might suggest that drinking TBEV-infected raw milk (colostrum) may result in TBE within a shorter incubation period than when TBE is associated with consumption of infected milk products (e.g., cheese). Therefore, short incubation should not be an exclusion criterion for the diagnosis of TBE, but in proven cases of TBEV infection, a short incubation period is likely a clue for alimentary transmission of TBEV.

Previously reported TBE outbreaks caused by alimentary transmission of TBEV lack definitive evidence of the virus having been present in milk or dairy products. However, in our study of 4 TBEV-infected persons, the source of infection was proven by direct demonstration of TBEV RNA and a corresponding virus load (concentration) in serum and milk samples from the goat whose milk was consumed. The outbreak described herein could have been avoided if the milk had been pasteurized or boiled before consumption or if the persons who became ill had been protected by vaccination, as was the fourth person who drank the TBEV-infected raw milk but did not become ill.

The increasingly fashionable natural lifestyle encourages the consumption of raw milk and products made of

unpasteurized milk. Even though alimentary transmission of TBEV is rare, the risk of such exposures could be reduced through education campaigns that encourage persons to consume only milk that has been boiled or pasteurized and only dairy products made from pasteurized milk. In addition, TBE vaccination, which effectively protects against tick bite–associated and dairy product–associated TBEV transmission, should be encouraged in areas where TBEV is highly endemic.

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Zombies—A Pop Culture Resource for Public Health Awareness

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Sitting at his laboratory bench, a scientist adds mutation after mutation to a strand of rabies virus RNA, unaware that in a few short days, an outbreak of this very mutation would destroy society as we know it. It could be called "Zombie Rabies," a moniker befitting of the next Hollywood blockbuster—or, in this case, a representation of the debate over whether a zombie apocalypse, manufactured by genetically modifying one or more diseases like rabies, could be more than just fiction. Fear of the unknown has long been a psychological driving force for curiosity, and the concept of a zombie apocalypse has become popular in modern society. This article explores the utility of zombies to capitalize on the benefits of spreading public health awareness through the use of relatable popular culture tools and scientific explanations for fictional phenomena.

Although zombies are currently an integral part of our popular culture, our morbid fascination with the walking dead spans several centuries. Historians and anthropologists trace the origin of zombies to the folklore of several tribes in western Africa, from Ghana to Nigeria (1,2). During the slave trade of the late 1500s through the 1800s, persons from these regions were spirited away from their homes to till the plantations of the Caribbean and the European colonies, bringing with them the voodoo culture of magic and spells. Among some academics, zombies in the New World were thought to be wretched, half-dead creatures that reflected the bondage African-born and Caribbean slaves suffered at the hands of their masters, working to the point of exhaustion in the plantation fields while having little to no agency (2). To this day, voodoo is prominent in western Africa, Haiti, New Orleans, and parts of the Caribbean Islands (1).

Haitian voodoo folklore recognizes a dual identity of *zombis*: one form of *zombi* is an ambulatory body without a living soul, and the other, lesser-known form is a soul wandering without a body (*2*). This severance of a body and its soul, known as zombification, is thought to occur when a sorcerer, or *boko,* performs a combination of dark magic spells on a person to kill, enslave, or inflict illness upon him (*3*). *Bokos* may also use poisonous powders in which frog or toad venom and tetrodotoxin, a powerful neurotoxin secreted by puffer fish that can trigger paralysis or death-like symptoms, could be primary ingredients. Which toxins are used in the zombie powders specifically, however, is still a matter of contention among academics (*4*). Once the sorcerer has split the body and soul, he stores the *ti-bon anj*, the manifestation of awareness and memory, in a special bottle. Inside the container, that part of the soul is known as the *zombi astral.* With the *zombi astral* in his possession, the sorcerer retains complete control of the victim's spiritually dead body, now known as the *zombi cadavre.* The *zombi cadavre* remains a slave to the will of the sorcerer through continued poisoning or spell work (*1*). In fact, the only way a *zombi* can be freed from its slavery is if the spell jar containing its *ti-bon anj* is broken, or if it ingests salt or meat. The latter would usually cause the *zombi* to hunt down and kill its master before finally returning to its family or its final rest as a corpse (*1,2*).

Although most cultures would consider the zombie to be a fictional creature, zombiism (i.e., being a zombie) is rather common in Haiti, with instances of people being reported dead by loved ones, only to be spotted fully reanimated and wandering around town several weeks to several years later. In Haitian and African culture, zombification is a punishable offense on the same order of severity as murder (*1*). A person who has been zombified, or transformed into a zombie, can have a blunt affect, dull gaze, and almost stuporous behavior, characterized by a lumbering gait and simple, repetitive vocalizations and movements. Most medical evaluations would characterize victims of zombification as having mental disorders such as catatonic schizophrenia (*1*). The aforementioned traits have been incorporated into the current interpretation of zombies found in modern film and media.

History of Zombies in the Media

Zombie folklore made its appearance in modern media in Das Cabinet des Dr. Caligari, a silent horror film directed by Robert Wiene, which debuted in Germany in 1921. The film's depiction of zombies paralleled Haitian lore: a sleepwalker under the control of another individual. The notion of a zombie was primarily defined by the control an individual had over another, and the main character in this film had the characteristic attributes of the early zombie: the unique lumbering gait, lack of higher cognitive ability, and obedience to another individual.

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Drawing inspiration from Richard Matheson's 1954 novel I Am Legend, George A. Romero spawned the more modern film manifestation of the undead zombie and the notion of a zombie apocalypse in The Night of the Living Dead. These zombies were the corporeal expression of strife, a mechanism to demonstrate rising social tension in response to a ruinous threat. Subsequent media have continued to use adaptations of Romero's zombie. The film adaptation for I Am Legend depicts humans who have undergone physiologic changes, developing intolerance for the sun and a unique form of communication while maintaining the ability to learn through mimicry and form social hierarchies. In the film 28 Days Later, infected humans transform into creatures characterized by preserved intellect and tremendously aggressive behavior. However, this expansion of aptitude is not uniform in modern media: Shaun of the Dead portrays zombies as very slow-moving, with incredible strength but no intelligence—they are fooled by normal humans who mimic their gait and groans. In addition, Juan of the Dead includes zombies dismissed by the Castro government as dissidents, and Warm Bodies depicts zombies as human protectors once they begin to transform back into humans. Land of the Dead revolves around the zombie siege of a noninfected gated community and sees the leader of the zombies gain class consciousness toward the end of the film. None of these interpretations are necessarily out of step with the use of zombie movies as a useful public health messaging tool.

Table. A comparison of zombies folklore and rabies epidemiology*

Characteristics	Zombies	Rabies
Susceptibility	Human infection requires fictional apocalyptic environment	Requires environment with infected animals, such as dogs or bats
Cause	Tyrant virus, other viruses, unknown pathogens	Mononegavirales
Virus transmission	Bites and scratches; unknown pathogen; spread human to human; 100% effectiveness	Bites; saliva infected with rabies virus; spread animal to human
Virulence	Victims die and become "walking dead"	Victims die and stay dead
Symptoms	Fever, chills, loss of hair and pigmentation, hobbling gait	Delirium, anxiety, stress, hallucinations, muscle spasms, convulsions
Control methods	Avoiding bites from existing zombies; intervention includes destroying brain of zombies	Avoiding bites from dogs and bats; postexposure prophylaxis
Exposure in popular culture		
Nonscientific media	Movies, books, television shows	Movies, books, television shows
Scientific media	Zombie websites, CDC, Nature	Academic journals, global health websites, NIH, CDC, Nature

*CDC, Centers for Disease Control and Prevention; NIH, National Institutes of Health.

Though most popular in film, zombies are present in other forms of media as well. They can be found in print, with novelizations such as Zombies for Zombies: Advice and Etiquette for the Living Dead and the popular comic book series Marvel Zombies. Increasingly, however, these flesh-eating monsters have found themselves in videogames, feasting upon unwary protagonists since the introduction of Zombie Zombie in 1984 (5). Zombies have since spread to the more general population in games such as Plants versus Zombies and Resident Evil, available on several different platforms of accessible technology.

A brief look through the history of the zombie's evolution within media unearths their progressing ability to serve as a vehicle to reach greater audiences. Frank Darabont's award-winning television adaptation of The Walking Dead comic books has proven that zombies maintain thematic prominence even when serving as the backdrop to a character-driven television drama. In his critically acclaimed novel World War Z: An Oral History of the Zombie War, Max Brooks explores social issues surrounding zombie apocalypse, such as the efficacy of government. These popular and varied manifestations of zombies elucidate the potential for a comprehensive dissemination of knowledge, from identifying traits indicating infection to explaining the significance of public health infrastructure. Zombies are a unique medium that allow for the audience's suspension of disbelief and for intellectual engagement.

Zombies and Parallels with Other Public Health Issues

Although zombies are certainly not the only favored supernatural creatures in modern times, they appear to be the best conduit to educate the layman about reemerging infectious diseases such as rabies. The current popular interpretation of vampires, for instance, has shifted away from the classic grotesque undead creature that voluntarily dines on the vein and sires new vampire progeny and instead has embraced the idea of vampirism as the paragon of human existence, alive or undead. The interpretation of zombies has been diverse, but at its core, zombiism remains an existence in which the victim has been stripped of any higher consciousness or agency. The reimagining of zombiism as a virulent, incurable disease makes it an effective analogy for understanding of and interest in other infectious diseases.

Zombie popularity may be a perfect opportunity to increase awareness of rabies. The most prominent resemblance between those afflicted with rabies and zombiism begins at the mouth; both ailments are primarily transmitted through biting (Table). While the pathogenesis for zombification is less consistent, rabies spreads through

infected saliva entering the body (*6*). In addition, victims indicate infected status with increased production of fluid from the mouth; in the case of rabies, increased salivation occurs to improve chances of transmission (*6*). Rabies control in practice may be similar to hypothetical control of zombie outbreaks. For example, in 2006, Chinese officials in the Yunnan province killed roughly 50,000 dogs in 5 days after an outbreak of rabies (*7*). This event sparked a great deal of controversy, and circumstances surrounding a similar rabies outbreak among dogs in Bali, Indonesia, in 2008 led to the primary alternative of mass vaccination. If a zombie apocalypse were to occur, surviving humans might not have the capacity for mass vaccination. The sole option may be to kill the undead for human survival; however, the ethics of destroying something that was once human might be called into question.

Additional physical characteristics of rabies and zombiism are similar (Figure). Once infected, victims display overall weakness and low-grade fever (*6*,*8*). In the case of zombiism, the advent of fever typically indicates the transition from human into zombie. When affected by rabies, human movement is irregular; muscle spasms and convulsions accompany numbness and loss of muscle function (*6*). Although their physical ability varies in media, the zombies we are familiar with generally have a distinctive, hobbling gait. Rabies causes difficulty swallowing because drinking causes spasms of the voice box; zombies largely lack the ability to produce any sound other than a deep groan, although they have been capable of speaking the word "brains" in classic zombie cinema.

Shared characteristics are not limited to physical attributes. A person with rabies will experience several changes in mental state, such as increased anxiety, stress, restlessness, delirium, abnormal behavior, and even hallucinations (*9*,*10*). Zombies will also typically display a limited level of cognitive function, with aggressive behaviors strengthening as cognitive function declines (*8*). However, there have been several exceptions. For example, in I Am Legend, the monsters were able to emulate a hunting trap made earlier by the protagonist. In the films 28 Days Later and 28 Weeks Later, the creatures had vastly amplified rage and slightly mitigated planning and judgment, while still mostly preserving other cognitive functions.

The numerous parallels between zombies and rabies, as well as other infectious diseases that are a threat to public health, enable the use of a popular media creature to promote the prevention and control of a public health problem. Pending specific training in public health or medicine, the layperson may gain substantial interest and understanding of rabies with our comparison and utility of zombies. In the media, protagonists always find a way to fight back against the zombies and try to maintain their survival. The attack on infectious diseases is similar to this fight against a new

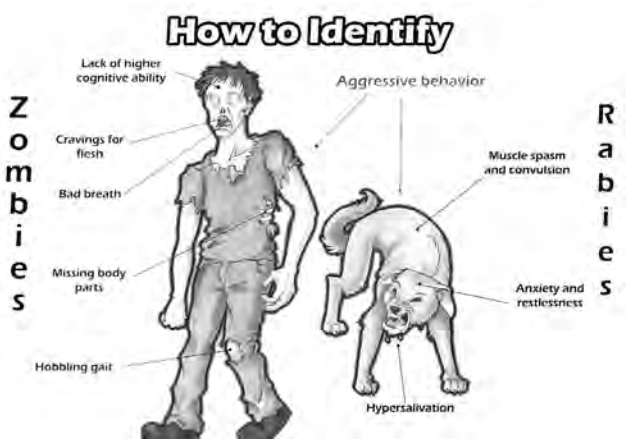

Figure. Comparison of physical characteristics of zombies and of an animal with rabies.

menace, in that new ways will be discovered along with those already known to prevent, treat, and control infections. The use of the zombie analogy can provide food for thought, thus providing inspiration for persons to be prepared for and prevent infectious disease outbreaks.

Zombie Psychology

Zombies may inspire fear within those who witness them in popular culture, and this fear can be compared with the same emotions that people might experience when they encounter the unknown. Some of the fears brought on by zombies include fear of brain dysfunction, fear of death, and feelings of hopelessness. Zombies, in turn, make these fears into something concrete, something we can reflect upon from a safe distance, as opposed to more active methods of facing our fears, such as high-risk activities like sky diving or bungee jumping. According to psychologists, watching *2*8 Days Later or navigating the characters of The Walking Dead through Telltale Games' deadly streets can be one way in which we, as psychologically bound humans, confront our fears and attempt to prepare for the possibility of our fears becoming reality. Although we would not go so far as to suggest the beauty of our fears in the face of hope, we should at least acknowledge the positive emotional effect that consideration of past success stories when handling rabies outbreaks, and infectious diseases in general, could have on our society should a zombie apocalypse occur. After all, a progressive society cannot be built upon feelings of fear and anxiety; what better way to build feelings of hope and community than drawing similarities between a seemingly unconquerable undead foe and a similar counterpart in the history of public health that was successfully managed (to a certain extent)?

The need for psychological preparation driven by fear can be a central motivation for community action,

of which public health outreach can take advantage (*11*). However, by using zombies in a slightly different manner, as an adaptable vehicle for instruction on prevention for rabies, and infectious diseases in general, we can introduce much of the psychology behind zombies that a blog post on disaster preparedness cannot. Much of the excitement and interest in our undead friends comes from such evaluations of shared human psyche between reality and a fictional postapocalyptic world; these may include, but are not limited to, slow degeneration of physical and mental health, ethical dilemmas, and issues of morality. For example, to generate empathy for patients having degenerative diseases, like rabies, Alzheimer's disease, or even cancer, parallels can be drawn to the sad realistic scenes of zombie films when the lone human (or zombie) survivor hangs on to dear life (or death) and sanity in the face of his encroaching demise. In addition, our former example of officials beating to death 50,000 potentially healthy dogs due to a rabies outbreak can be juxtaposed to the Kantian ethics behind killing potentially uninfected humans (for safety reasons, of course), who have just been bitten but still retain all of their cognitive functions. Even the morality of quarantining hundreds of thousands of humans citywide, sometimes even along racial and ethnic boundaries, can be more engaging when discussed within both contexts of rabies and that of zombies.

Discussion

Within the past couple of years, we have seen zombies gain traction not just in media, but also in the field of public health. Zombies entered the health circuit in mid-2011 when, after the Tohoku Earthquake in Japan in early March of that year, the Centers for Disease Control and Prevention (CDC) opened up a disaster forum on the microblogging site Twitter. In it, the CDC asked members of the general public what sorts of emergencies they were prepared for and what tactics they were using to ensure disaster preparedness. Several persons showed interest in preparing for a catastrophic zombie attack. These responses were most likely meant to be facetious, but they demonstrated the prominence of zombies in popular culture and their utility for drawing attention to health issues. As a result, Dr Ali S. Khan and his colleagues wrote a column on the Public Health Matters blog of the CDC Web site (*11*). They used zombies as the hook to draw readers into an article detailing how best to prepare for a zombie apocalypse and, by extension, any kind of disaster, be it natural or manmade.

The popularity of the CDC's tongue-in-cheek zombie apocalypse article prompted other organizations to create their own, shedding light not only on disaster preparedness but also the ethics of zombie killing. Among them is an op-ed piece written by Daniel O'Connor of Johns Hopkins University's Berman Institute of Bioethics (*12*). In the article, O'Connor outlined several bioethical guidelines to consider if one found oneself in a community that had a zombie infestation. Some of the guidelines explain the threshold at which a person can no longer be considered a human (e.g., being bitten by a zombie) and when and how to kill a zombie in a morally acceptable manner. Other guidelines underscored the importance of minimizing the risk-benefit ratio for as many people as possible when devising antizombie strategies, as well as the importance of community engagement. The most effective strategies incorporate the diverse voices of the community rather than relying solely on the judgment of the military, politicians, and health experts (*13*). Many of these bioethical guidelines come into play when promoting public health intervention strategies for several other diseases and health concerns, but O'Connor's article also subtly underscores the frailty of such guidelines in the face of disasters that threaten to destabilize health infrastructure. The article points toward a glaring need to ensure that the standards of public health are maintained even in the event of an apocalyptic pandemic event.

There has been speculation on whether a virus akin to rabies could become a threat similar to that of zombification. Authors of a National Geographic article titled "Zombie Virus" Possible via Rabies-Flu Hybrid? shed some light on what they believe to be an irrational fear of such a predicament (*14*). First, there is a mechanistic barrier to creating the perfect zombie virus from the rabies virus. Contrary to the immediate onset of zombification, the onset of rabies within the body is about 10 days to 1 year for incubation. In addition, there is a genetic barrier to induce the symptoms exhibited in zombification within the genetic code of other viruses; it is scientifically unheard of for 2 viruses to borrow traits or fuse (*14*). Despite the availability of cutting-edge genetic engineering that could combine the airborne potential from influenza, personality changes from measles, fever and delirium from encephalitis, and internal bleeding/necrosis from the Ebola virus with that of the rabies virus, little certainty exists that such a virus could be viable in vivo and still leave its host alive.

If a cocktail of deadly virus could not even mimic the traits of a zombie, is there any other microorganism out there that could? In fact, this answer is closer than one would expect. Simple misfolding of proteins in the brain can lead to the creation of a whole family of these anomalies, called prions. Prions can ultimately lead to brain disease, encephalopathy, which may result in personality changes, loss of cognitive function, and muscle twitching, all highly characteristic of a potential zombie. Fortunately, current scientific evidence reveals prions as incapable of causing widespread epidemics, and with an incubation period of 12–18 months before death, prions

do not seem much of a threat to be compared with a zombie apocalypse.

The government might also be a barrier to a zombie outbreak, by isolating and controlling the source of such infections from circulating worldwide. Still, some may argue that governmental response to a zombie apocalypse could lead to discrimination against zombies as a minority population. We assume that much of postapocalyptic society relies on the often untrue depiction, and in some cases even satire, of nonfunctioning or ill-functioning government for entertainment value. Still, when we are dealing with disease outbreaks beyond our understanding, we must first develop a capable society that can effectively deal with outbreak control and civic problems before mentioning any possibility of a cure. For now, we can at least rest in peace without worrying that a zombie epidemic is just around the corner.

Conclusions

Zombies can be used as a powerful tool for increasing awareness of issues of public health significance. The popularity of the CDC piece on preparing for a zombie apocalypse has been instrumental in teaching how to prepare for disasters like the Tohoku Earthquake in Japan. We propose continuing these efforts, building on the popularity of zombies to increase public health awareness in the general public, and explore additional issues that may have not been considered in the past, such as infection control, mental health issues, ethics of disease, and bioterrorism potential. These issues can be explored by taking advantage of various forms of media, including 1) distributing informational pamphlets, books, and other printed media explaining the similarities between zombie infestations and lesser–known outbreaks and how to protect oneself and others; 2) creating satirical or dramatic public service announcements to promote defensive community strategies against infectious disease outbreaks by using zombiism as an analogy; 3) using interactive games, computer programs, and smartphone applications to enable the public to safely experience the natural progression of real epidemics on different levels of aggregation (e.g., individuals, communities, policymakers, medical and public health officials, both infected and uninfected), with and without intervention strategies such as vaccination, quarantine, or extermination; and 4) and facilitating the creation of clubs, societies, blogs, and even magazine articles across age ranges, academic institutions, and internet interest groups to share the excitement of applying what we can learn about zombies to more applicable avenues of life, such as public health and epidemiology. We must also consider the possibility–no matter how remote–that zombies could very well be replaced by other popular culture icons in the future. To that end, we must continue to adapt and use these pop culture tools to increase interest in and awareness of notable public health issues affecting the world.

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Plasmodium falciparum with Multidrug Resistance 1 Gene Duplications, Senegal

To the Editor: Amplification and overexpression of the *Plasmodium falciparum* multidrug resistance 1 gene (*Pfmdr1*) have been associated with mefloquine resistance in *P. falciparum* malaria in Asia (*1*). Amplification of *Pfmdr1* in Africa has occurred rarely. Only 12 isolates with ≥2 copies of *Pfmdr1* were identified in Africa during 1993–2012: 3 in Côte d'Ivoire (*2*,*3*), 1 in Burkina Faso (*3*), 1 in Togo (*3*), 3 in eastern Sudan (*4*), 2 in Kenya (*5*,*6*), and 1 Senegal (*7*). Another isolate was obtained in a patient from Benin who did not respond clinically to mefloquine treatment (*8*). *Pfmdr1* amplification has not been found in samples collected either before or after treatment for recurring *P. falciparum* infection in Africa in many studies.

In Dakar, Senegal, and its surrounding suburbs, malaria is transmitted with spatial heterogeneity to the human mosquito bite rate, which ranged from 0.1 to 250 bites per person per night during the rainy seasons of 2007–2010. *P. falciparum* isolates from patients with malaria who lived in Dakar (>80%) and the surrounding area and did not travel during the previous month were obtained during the rainy seasons of October 2009–January 2010 (172 patients, 42% female) and August 2010–January 2011 (129 patients, 38% female). Informed verbal consent from the patients and/or their parents/guardians was obtained before blood collection; the study was approved by the ethical committee of the Hôpital Principal de Dakar.

Of the 301 patients, 54% were recruited from the emergency department during each of the 2 seasons; other patients were recruited from the intensive care unit (18% during October 2009–January 2010 and 20% during August 2010–January 2011), pediatric department (9% and 5%), and other units (19% and 21%). No significant differences were found between the 2 seasons for parasitemia ($p = 0.160$), sex ratio ($p = 0.446$), living area ($p = 0.651$), or hospital admission status ($p = 0.567$). Information on antimalarial treatment before admission was not available.

We analyzed the *Pfmdr1* copy number for the 301 isolates by using TaqMan real-time PCR as described (*7*); 167 isolates were successfully evaluated by using the 72-h histidine-rich protein 2 test as described (*9*). *P. falciparum* clones 3D7 (1 *Pfmdr* copy) and W2 (3 *Pfmdr1* copies) were used as controls for the determination of the *Pfmdr1* copy number and for the validation of the batches of plates used in the susceptibility tests.

A total of 9 isolates with 2 *Pfmdr1* copies were identified, 1 collected during the 2009–2010 season and 8 collected during the 2010–2011 season. This finding reflects a statistically significant 10-fold increase in frequency of isolates from 1 season to the next ($p = 0.0057$ by Fisher exact test). All of the isolates with duplicated copies had 1 allelic family for each of the 3 genes (*msp1*, *msp2*, and *glurp*), confirming that these infections were single and not mixed.

We did not find an association between *Pfmdr1* copy number and the 50% inhibitory concentration values for mefloquine ($p = 0.345$), monodesethylamodiaquine ($p = 0.729$), lumefantrine ($p = 0.314$) and chloroquine ($p = 0.579$), but a significant association was found for dihydroartemisinin ($p = 0.003$). Odds ratio for in vitro reduced susceptibility to dihydroartemisinin associated with 2 *Pfmdr1* copies was 1.4. Although the number of isolates with 2 *Pfmdr1* copies we obtained is small, these data are consistent with previous reports on *Pfmdr1* copies and in vitro responses to artemisinin derivatives (*10*).

The role of the amplification of *Pfmdr1* in *P. falciparum* resistance to antimalarial drugs in Africa is debated. In our study, we identified as many samples with multiple *Pfmdr1* copies (9) as had been identified during the previous 19 years. The 9 patients from whom these isolates were collected were successfully treated with quinine. One study found clinical treatment failure for mefloquine in Africa was associated with in vitro resistance and amplification of *Pfmdr1* (*8*). In addition, amodiaquine resistance is not related to the amplification of *Pfmdr1* (*6*). In Sudan, 3 isolates with 2 *Pfmdr1* copies were identified in patients before and after treatment with artemether-lumefantrine during the 28-day follow-up period, but these patients were classified as having adequate clinical and parasitologic responses (*4*).

The increased prevalence of *Pfmdr1* duplication in *P. falciparum* isolates from patients in Dakar within a 2-year period is cause for concern and vigilance. The presence of these duplicated *Pfmdr1* copies could be associated with rare clinical failures of *P. falciparum* infections to respond to mefloquine treatment or artemisinin-based combination therapy. However, our findings highlight the need for active surveillance of the prevalence of *Pfmdr1* duplication in *P. falciparum* isolates and for ex vivo and in vivo studies in Senegal and in other parts of Africa.

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Atypical Erythema Migrans in Patients with PCR-Positive Lyme Disease

To the Editor: The best diagnostic sign in patients with early Lyme disease is a skin lesion, erythema migrans (EM). However this sign may not occur or be recognized in 30% of cases (*1*). Furthermore, the EM rash may not display a classic bull's-eye (ring-within-a-ring) appearance, a fact that may be underappreciated (*2*,*3*). Some studies noted uncharacteristic variants of EM in 25%–30% of cases (*4*–*7*). One study reported the rash to be uniformly red in 60% of cases (*6*). Other atypical variants of EM are a blue-red appearance and, occasionally, a vesicular central region (*4*,*5*). We describe the occurrence of atypical EM in patients with microbiologically proven *Borrelia burgdorferi* infection.

During spring and summer 2009, a total of 29 patients with classic or possible EM and suspected Lyme disease were referred by primary care physicians for an ongoing prospective study. Laboratory methods have been described (*8*). The patients were >18 years of age and lived in suburban Baltimore, Maryland, USA, where Lyme disease is endemic. All patients had extracutaneous manifestations (e.g., virus-like symptoms). Fourteen patients met laboratory criteria for study analysis: 1) positive PCR at the initial study visit, detected by a *B. burgdorferi*–specific nucleic acid–enhancing PCR method on a 1.25-mL whole blood sample (*8*), and 2) evidence of *B. burgdorferi* exposure by the 2-tiered antibody test at the initial or posttreatment visit. Other entry criteria were a rash >5 cm and symptoms compatible with early Lyme disease (*1*); exclusion criteria were certain preexisting medical conditions (*8*).

A panel of experienced specialists, including dermatologists, were shown photographs of the patients' skin lesions and asked if they would expect the average primary care physician to diagnose the lesions as EM. To avoid bias, PCR and serologic test results were withheld from the specialists and they were asked to categorize lesions by characteristics common to target-like and non–target-like lesions. Lesions with the classic bull's-eye appearance, with central clearing and peripheral erythema, were classified as classic EM; those with uniform red or red-blue or other appearance and lacking central clearing were classified as possible atypical EM. If any lesion of a multiple lesion set was classic in appearance, we categorized the rash as classic EM. Of the 14 patients with positive PCR, 10 had nonclassic EM (Figure) and 4 had classic, target-like EM. Atypical rashes varied from those close to classic EM to those resembling

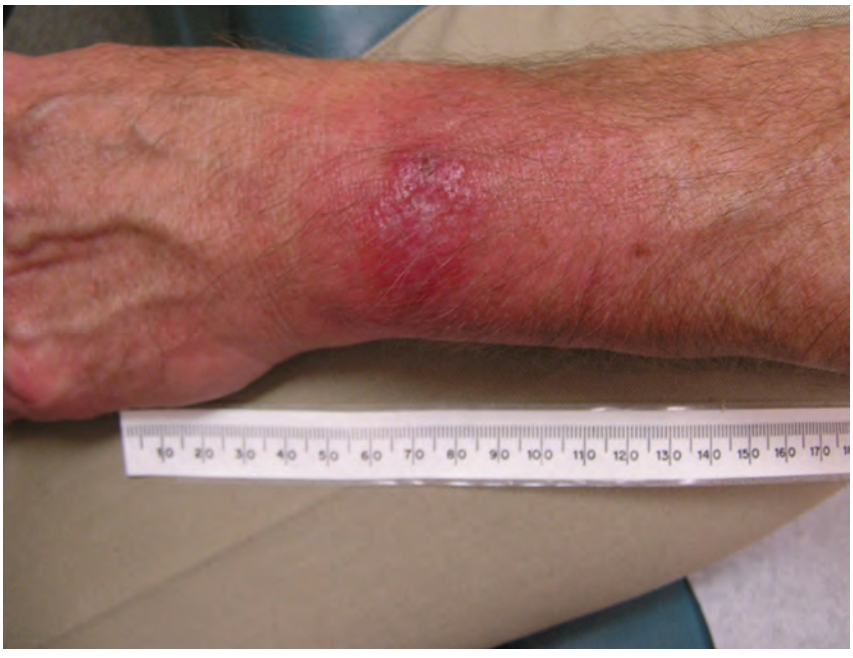

Figure. Atypical erythema migrans lesion on a patient with PCR-positive result for *Borrelia burgdorferi* infection. The rash was not considered typical because it lacked central clearing and peripheral erythema. The differential diagnosis included a contact dermatitis and arthropod bite. At the initial examination, this patient was seronegative for *B. burgdorferi* by 2-tiered criteria. Three weeks after therapy, the patient had positive results for ELISA and IgM Western blot and negative results for IgG Western blot, providing evidence of seroevolution (i.e., increasing antibody titer and/or increase in band intensity or appearance of new antigen bands to *B. burgdorferi*).

lesions more common in other conditions (e.g., insect or spider bites) and, consequently, prone to misdiagnosis.

Depending on the appearance of an atypical rash, the differential diagnosis could include contact dermatitis, arthropod bite, or, in cases with annular lesions, fixed drug eruptions, granuloma annulare, cellulitis, dermatophytosis, or systemic lupus erythematosus (*5*). In addition, a diagnosis can be more challenging when there are multiple skin lesions rather than a single lesion and in a pattern unfamiliar to a general practitioner.

Multiple textbooks and websites have featured pictures of EM as a bull's-eye lesion (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0796-Techapp.pdf). This emphasis on target-like lesions may have inadvertently contributed to an underappreciation for atypical skin lesions caused by Lyme disease. Nevertheless, physician recognition of Lyme disease–associated EM is essential because current approved laboratory tests may not identify *B. burgdorferi* in the first few weeks of infection (*8*), when an accurate diagnosis can lead to early curative therapy.

Separate studies found different percentages of atypical Lyme disease–associated rashes (*3*,*4*,*9*); each was lower than the percentage found in our study. Our study has several limitations: it encompassed only 1 recruitment season, 1 geographic site, and a small number of patients. The sensitivity of PCR for blood specimens is improving (*8*); however, PCR may have missed some acute cases in our study for reasons cited below. Therefore, these patients should not obligatorily be considered as representative of all acute Lyme disease patients.

Our study results serve as an impetus for studying more patients with systemic and nonsystemic signs and symptoms over multiple seasons and geographic areas and for including PCR analysis of skin lesions in future studies. PCR of skin biopsy samples may provide insight as to whether a negative blood PCR is the result of infection with a skin-restricted strain (*10*) in patients in whom bacterial dissemination is not expected or a result of low copy number of *B. burgdorferi* in the blood sample.

Our results serve as a reminder that patients with early Lyme disease may have an atypical rash, not the classic (textbook) bull's-eye lesion. Close observation and a detailed history of whether the rash is enlarging, has enlarged, or is spreading should be part of the consideration of the diagnosis. Observation for extracutaneous signs of early infection, such as cranial seventh nerve palsy (Bell's palsy) or meningitis, is also essential.

In summary, the EM rash of Lyme disease can have an atypical appearance. Thus, clinicians should consider Lyme disease in the differential diagnosis of patients who have a rash that may not be classic EM and who have been in areas where Lyme disease occurs.

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Brucellosis in Guangdong Province, People's Republic of China, 2005–2010

To the Editor: Brucellosis is one of the most prevalent zoonotic diseases in the world. It is principally an animal disease, but globally, >500,000 human cases are reported each year (*1*). Transmission to humans occurs primarily through contact with infected animals and consumption of contaminated food (*2*,*3*). Persons with occupational exposure are at highest risk for brucellosis, in particular those performing husbandry activities, butchering, and livestock trading (*4*,*5*).

Although brucellosis has been eradicated from many industrialized countries, new foci of disease continually appear, particularly in parts of Asia (*6*–*8*). In China, 160,214 brucellosis cases were reported during 2005–2010; 90% of them occurred in 6 northern agricultural provinces: Neimenggu, Shanxi, Heilongjiang, Hebei, Jilin, and Shaanxi. Livestock, such as goats, cattle, and pigs, are the main infectious source. However, factors such as the rapid movement of people from northern to southern China, increased livestock trading, and lack of livestock quarantine mean that infected livestock or their products readily traverse provincial borders and transmit disease to persons who have no direct contact with livestock.

With an illness rate of <0.01 cases/100,000 population, Guangdong Province in southern China is one of the areas in China with the lowest incidence of brucellosis (*9*), but incidence is increasing. During 1955–2004, Guangdong Province recorded 51 confirmed cases of brucellosis; however, during 2005–2010, 112 cases were reported. All reported cases had typical clinical characteristics, including undulant fever, night sweats, chills, and weakness; some cases were associated with encephalitis, meningitis, and arthritis. Of the 112 reported cases during 2005–2010, 105 were laboratory confirmed: 61 by culture (55 from blood culture, 3 from bone marrow, and 1 each from joint fluid, cerebrospinal fluid, and a vertebrae disc abscess); and 44 by serum agglutination test (SAT; single titer >400). The male:female ratio among these patients was 66:46. The age ranges were similar by sex; male patients were 18–71 (median 47) years of age, and female patients were 20–70 (median 43) years of age.

The first 3 cases of brucellosis in 2005 were reported in Shenzhen in Guangdong Province. One case was culture confirmed by clinical laboratory, and the isolate was identified as *Brucella melitensis* biovar 3 by SAT and phage biotyping. The other 2 cases were in dairy farm workers; their infections were laboratory confirmed by SAT but could not be identified by biovar. Since 2005, more cities in Guangdong have reported brucellosis cases (Figure, Appendix, wwwnc.cdc.gov/EID/article/19/5/12-0146-F1.htm). The Pearl River Delta region reported 100 cases: 48 in Guangzhou, 27 in Shenzhen, 7 in Zhongshan, 6 in Foshan, 6 in Jiangmen, 4 in Zhuhai, and 2 in Dongguan. Only 12 cases were reported from undeveloped rural areas in Guangdong: 5 in Zhaoqing, 2 in Yangjiang, and 1 each in Huizhou, Qingyuan, Meizhou, Maoming, and Yunfu.

A total of 42 *Brucella* isolates were cultured during 2005–2009, and all were identified as *B. melitensis* biovar 3. However, of 19 *Brucella* isolates cultured during 2010, a total of 13 were identified as *B. melitensis* biovar 3, 4 as *B. melitensis* biovar 1, and 2 as *B. suis* biovar 3. These results indicate a shift in species and biovar for *Brucella* spp. circulating in China.

We conducted a retrospective epidemiologic investigation of the 112 brucellosis cases reported during 2005–2010 to identify the vehicles and sources of infection. Among the cases identified, 33 (29.46%) patients had occupational exposure history: 13 were pig or goat butchers, 12 dairy farmers, 5 animal market workers in charge of leading the animals to and from transportation, and 3 mutton and pork sellers in wet markets. The remaining 79 (70.54%) cases were in patients who denied having contact with living animals. Among these patients were retired persons, housekeeping matrons, teachers, doctors, white collar workers, and the unemployed. However, 17 of these patients recalled having purchased or handled goat placenta to be prepared for home consumption or having eaten goat products through barbequing or hot pot. The other 62 could not remember if they had contacted with livestock or their products. These findings indicate that nonoccupational exposure may pose a risk for brucellosis infection among persons who handle fresh meat and meat products for home cooking.

In conclusion, Guangdong Province has become an emerging foci for brucellosis in China. The species and biovars of *Brucella* spp. circulating in this region are changing, and many persons are infected by nonoccupational exposure. Measures need to be taken by central and provincial governments to address these issues and prevent epidemics of brucellosis in humans.

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Cutaneous *Mycobacterium shigaense* Infection in Immunocompetent Woman, China

To the Editor: *Mycobacterium shigaense* is a novel, slow-growing, scotochromogenic mycobacterium (*1*), initially reported in 2012 as an opportunistic pathogen isolated from skin biopsy samples from a patient with a history of Hodgkin disease and severe cellular immunodeficiency. We describe the identification of this species in a chronic cutaneous infection in an immunocompetent woman.

A 56-year-old woman was admitted to our inpatient department in August 2011 with reddish papules, nodules, plaques, and scars on her face and neck (Figure, panel A) that had developed over >1 year, starting in June 2010. Initially, a few small papules appeared on her face; the primary papules gradually enlarged, spreading to the neck and developing into nodules, plaque, partly purulent lesions, and sometimes fistulas associated with moderate pain and scarring. The patient reported no history of trauma or surgical procedure and could not recall any potential inducement of the lesions or previous receipt of immunosuppressant therapy.

On physical examination, the patient was thin, with normal vital signs. Results of routine laboratory tests were unremarkable. Cephalic and cervical radiographs and computed tomography scans revealed lymphadenectasis of the neck only; a purified protein derivative test showed an erythema ≈8 mm in diameter on the forearm. Results of testing for HIV and human T-lymphotropic virus 1 antibody detection tests were negative. Cell-mediated immunity levels were detected by flow cytometry of peripheral blood cells; cell counts for CD3+, CD4+, CD8+ T-cells, and T-cell receptors and for CD19+ B-cells were within reference ranges.

Skin samples were collected from the face and neck of the patient and subjected to histopathologic examination, smear testing, and culture. Histopathologic examination showed a hyperplastic epidermis and noncaseating granulomatous infiltrates of lymphocytes, histiocytes, and multinucleate giant cells in the dermis (Figure, panel B). Results of Ziehl-Neelsen staining of smears for acid-fast bacilli and periodic acid–Schiff staining for fungi were negative; fungal and other bacterial cultures were sterile. Samples streaked on Löwenstein-Jensen medium at 32°C and 37°C for 4 weeks formed smooth, creamy, yolk yellow colonies (Figure, panel C); however, such colonies did not grow at 25°C and 45°C. Ziehl-Neelsen staining of samples from the colonies revealed acid-fast bacilli (Figure, panel D). Infection with a mycobacterium was suspected on the basis of these results.

The isolated bacilli were subjected to PCR restriction fragment-length polymorphism analysis and sequencing of the mycobacterial *hsp65* gene to identify the bacteria and strain (http://app.chuv.ch/prasite/index.html) (*2*,*3*). However, a match for the restriction pattern or the sequence of the isolated organism and *Mycobacterium* spp. was not found. Therefore, complete DNA sequences of the 16S rRNA, *hsp65*, and *rpoB* genes and the 16S–23S rRNA internal transcribed spacer (ITS) region were determined by using primers and PCR protocols described previously (*4*–*7*). The sequences of 16S rRNA, *rpoB* and the 16S–23S rRNA ITS region were identical to those of the nontuberculous mycobacterium species *M. shigaense*; the *hsp65* gene showed 94% similarity (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1022-Techapp1.pdf).

Results of biochemical tests of the isolate were positive for *Mycobacterium* spp. by a 2-week culture on MacConkey agar and a heat catalase test but

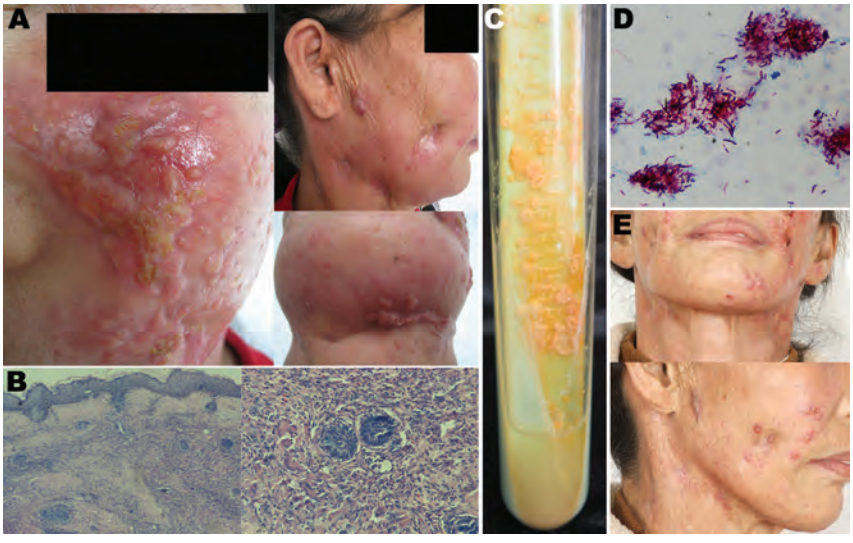

Figure. Cutaneous *Mycobacterium shigaense* infection in a 56-year-old Immunocompetent woman, China. A) Plaques, scars with scabbing, nodules, and concave scars on the face and neck and papules and scarring on the submaxilla. B) Histopathologic results, showing hyperplastic epidermis and infiltration with lymphocytes, neutrophilic leukocytes, multinuclear giant cells, and epithelioid cells in the dermis. C) Samples streaked on Löwenstein–Jensen medium at 32°C formed smooth, yolk yellow creamy colonies. D) Ziehl–Neelsen staining of bacilli from the colonies that tested positive. E) Visible improvement of lesions after 4 months of treatment.

weakly positive by 3-day arylsulfatase and 2-week catalase testing (*8*). Results of nitrate reduction, semiquantitative catalase, growth in 5% NaCl medium, urease, and Tween 80 hydrolysis testing were negative. In vitro drug susceptibility was investigated by using the microdilution method, according to Clinical and Laboratory Standards Institute guidelines (*9*). The isolate was susceptible to moxifloxacin, amikacin, clarithromycin, rifampin, ethambutol, streptomycin, and ofloxacin.

The patient was treated with orally administered rifampin (450 mg 1×/d), moxifloxacin (400 mg 1×/d), and clarithromycin (500 mg 2×/d) for 6 months. The lesions subsided, leaving hyperplastic and atrophic scars (Figure, panel E). New nodules did not recur, and no notable side effects were found.

In general, culture-based identification methods using biochemical tests are slow and inadequate in differentiating species of mycobacteria. Laboratory methods with better performance, such as genetic investigations using nucleic acid amplification and sequencing, are increasingly used for identification. In this case, the complete gene sequences of 16S rRNA, 16S–23S rRNA ITS region, *rpoB,* and *hsp65* of the isolate were used to find the most consistent and highest scoring match across all 4 loci in GenBank (www.ncbi.nlm.nih.gov/genbank).

In conclusion, our results strongly suggest that this chronic cutaneous infection in an immunocompetent patient was caused by *M. shigaense*. Our observations provide further evidence that this species should be classified as a nontuberculous mycobaterium that can cause disease in immunocompromised and immunocompetent patients. The isolate we identified was classified as clinically pathogenic and not an environmentally contaminating strain because it was isolated from multiple lesions and at different times. The lesions improved after treatment with clarithromycin and moxifloxacin, and the bacterium was not detectable thereafter.

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Infectious Disease Surveillance by Medical Examiners and Coroners

To the Editor: Medical examiners and coroners (ME/C) investigate ≈20% of all deaths in the United States (*1*); these include persons who die outside the health care system or die precipitously without a confirmed diagnosis. Surveillance through ME/C offices for unexplained deaths that might have infectious causes can serve as a sentinel system to identify new agents, identify notifiable diseases missed by traditional surveillance systems, recognize unique signs and symptoms of known pathogens, and detect bioterrorism (*1*). This surveillance model, called Med-X, is based on standards for autopsy performance, diagnostic testing, and public health reporting and is currently being performed locally in a small number of offices.

To assess more widely the capacity of ME/C offices to conduct infectious disease surveillance, the National Association of Medical Examiners distributed an Internet-based questionnaire to 155 ME/C offices in the United States that serve populations >300,000; the questionnaires were completed during August–September 2009. Survey questions addressed interest in and physical, personnel, and logistical capacities for conducting surveillance for deaths that could have resulted from infectious diseases. Because many infections can be transmitted during autopsy, specific biosafety features for the autopsy suite were also assessed.

The ME/C offices that responded (68/155) are responsible for 59% of the population served by the target ME/C offices and, on average, perform autopsies on 33% (range 12%–80%) of their cases. Most of the responding offices were the principal office for the area, which was primarily at the county or parish level. Of the responding offices, 97% indicated an interest in a medical examiner–based surveillance system for infectious diseases; 13% currently identify and report cases through the Med-X system. Almost half of the respondents noted some Biosafety Level 3 features in their facilities, including negative pressure ventilation, double-door entry into autopsy suites, or appropriate air exchange and ventilation systems. With respect to current capabilities and practices of surveillance of infectious diseases, most respondents had optimal databases that contained complete and searchable data that included circumstances of death narrative, autopsy findings, and laboratory results. Most offices also had established practices of identifying infectious diseases and of reporting to local or state health departments notifiable and nonnotifiable diseases.

The most often cited barriers to participation in ME/C infectious disease surveillance were funding and resources (85%), lack of supplies (76%), insufficient laboratory testing capability (69%), and personnel requirements (63%). These factors all relate primarily to the subsequent autopsies resulting from the surveillance. With respect to current autopsy practices, survey results suggest that inadequate usage of personal protective equipment (6%), lack of autopsy suites with negative pressure (21%), and inadequate required vaccinations (e.g., hepatitis B) for pathologists (40%) are areas where improvement is needed.

During the past few decades, several diseases of public health importance, including new or emerging infectious diseases, have been recognized and identified through the collaborative efforts of public health partners and medical examiners, performance of autopsies, and subsequent postmortem diagnostic testing (*2*–*4*). The findings from this survey suggest that interest and potential exist for the establishment of an enhanced national ME/C-based surveillance system for novel or emerging infectious diseases and bioterrorism. A surveillance protocol is already available for distribution (*5*). Although survey respondents showed high interest in such a system, this result may be an overestimation because of the offices targeted and the low overall response rate. Addressing existing barriers, including funding and infrastructure deficiencies, may increase participation in such a national surveillance system. Development of a national surveillance system of this type would require fulfilling recently identified steps needed to strengthen the competency of national death investigation systems (*6*), establishment of uniform statewide and interstate standards of operation such as those outlined in the National Association of Medical Examiners accreditation checklist (*7*), consolidation of smaller offices, regionalization of services, and standardization of staff training.

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Multidrug-Resistant *Acinetobacter baumannii* Clone, France

To the Editor: *Acinetobacter baumannii* is an opportunistic pathogen that is a source of nosocomial infections, mostly pneumonia (*1*). Treatment of infections caused by *A. baumannii* is becoming a serious clinical concern as this microorganism becomes increasingly resistant to multiple antimicrobial drugs (*2*). *A. baumannii* resistance to carbapenems is mostly associated with production of carbapenem-hydrolyzing class D β-lactamases and metallo-β-lactamases (*2*). New Delhi metallo-β-lactamase 1 (NDM-1) is one of the most recently discovered metallo-β-lactamases among various gram-negative species, including *A. baumannii* (*3*). We recently reported the recovery of NDM-1–producing *A. baumannii* isolates throughout Europe (*4*). In that study, the genetic background of several strains was identified and corresponded to sequence types (STs) 1, 25 and 85. The ST85 clone was isolated in France from 2 patients previously hospitalized in Algeria (*4*,*5*).

The present study was initiated by the recent isolation of 6 more NDM-1–producing *A. baumannii* linked with North Africa. To determine the extent of spread of this organism from Africa to France, we genetically analyzed 8 other NDM-1-producing *A. baumannii* isolates collected from different towns in France during 2011–2012. Of these 8 isolates, 6 were from patients previously hospitalized in different cities in Algeria (including Algiers, Setif, Constantine, and Tlemcen), 1 from a patient previously hospitalized in Tunisia, and 1 from a patient previously hospitalized in Egypt. These 8 isolates came from 2 clinical samples (blood cultures and wound) from 6 screening rectal swab samples collected at the time of hospital admission (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1618-Techapp1.pdf). Because the 8 samples were recovered from 5 hospitals, nosocomial acquisition can be ruled out.

The isolates were identified by 16S rRNA gene sequencing. Susceptibility testing was performed by disk diffusion (Sanofi-Diagnostic Pasteur, Marnes La Coquette, France) and interpreted according to updated Clinical and Laboratory Standards Institute guidelines (*6*). The MICs of β-lactams (imipenem, meropenem and doripenem) were determined by the Etest technique (AB bioMérieux, Solna, Sweden) according to the manufacturer's recommendations. All isolates were resistant to β-lactams, including all carbapenems (MICs >32mg/L). The isolates were also resistant to fluoroquinolones, gentamicin, sulfonamides, and chloramphenicol but susceptible to amikacin, netilmicin, rifampin, tetracycline, and tigecycline according to Clinical and Laboratory Standards Institute guidelines (*6*) and colistin according to European Committee on Antimicrobial Susceptibility Testing guidelines (www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v1.3_pdf.pdf).

The production of metallo-β-lactamases was suspected by use of a combined disk test, based on the inhibition of the metallo-β-lactamase activity by EDTA as described (*4*). All isolates were positive for production of metallo-β-lactamases.

For all 8 isolates, PCRs aimed at detecting carbapenemase genes, using primers described elsewhere (*7*), followed by sequencing, led to identification of the $bla_{\text{NDM-1}}$ gene. The isolates also carried a naturally-occurring $bla_{\text{OXA-51}}$-like gene, namely $bla_{\text{OXA-94}}$ (online Technical Appendix). The $bla_{\text{OXA-51-like}}$ β-lactamase confers a low level of resistance to carbapenems.

Genotypic comparison was performed by multilocus sequence typing as described (*8*) and by repetitive extragenic palindromic sequence-based PCR by using the DiversiLab system (bioMérieux, La Balme-les-Grottes, France) according to the manufacturer's instructions. The genomic pattern of all isolates was identical (Figure). Further multilocus sequence typing indicated that all isolates belonged to ST85. This ST was identified in Greece during a nationwide study that focused on carbapenem resistance in clinical isolates of *A. baumannii* and

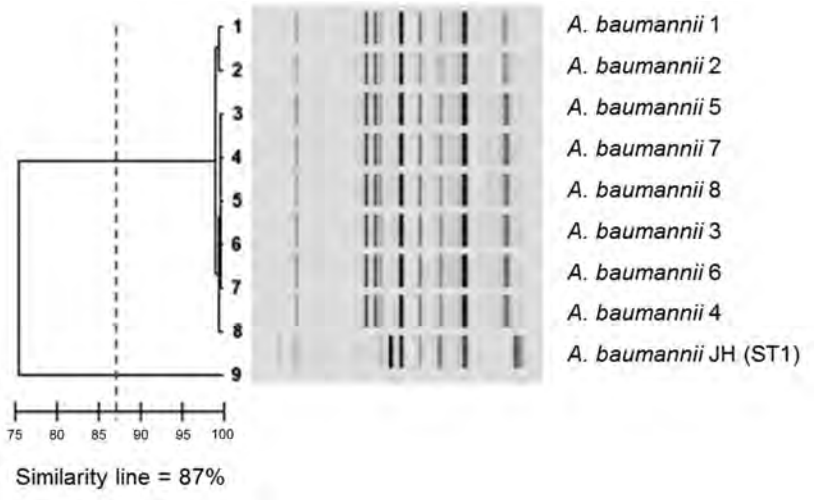

Figure. Results of Diversilab system (bioMérieux, La Balme-les-Grottes, France) analysis of *Acinetobacter baumannii* isolates. Similarity line shows the cutoff that separates the different clones.

identified mainly carbapenem-hydrolyzing carbapenemase OXA-58 (*9*).

Recently, we showed that the bla_{NDM-1} gene was carried by a composite transposon bracketed by 2 copies of IS*Aba125* in *A. baumannii* (*10*). Cloning and sequencing of the genetic context of the bla_{NDM-1} in the first isolate showed that transposon Tn*125* was truncated at its 3′-end extremity by insertion sequence IS*Aba14*, giving rise to a truncated Tn*125* (ΔTn*125*). PCR mapping of all isolates showed that they possessed this truncated isoform of Tn*125*, which was therefore probably no longer functional.

The identification of several clinical *A. baumannii* isolates that possessed the bla_{NDM-1} gene and originated from North Africa, with no obvious link to the Indian subcontinent, strongly suggests that 1 NDM-producing *A. baumannii* clone is probably widespread in North Africa and that it might now act as a reservoir for NDM-1. This finding might indicate that control of spread of multidrug-resistant *A. baumannii* would have a primary role in controlling spread of NDM-1.

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Genomic Analysis of *Salmonella enterica* Serovar Typhimurium **Definitive Phage** Type 104

To the Editor: *Salmonella enterica* is among the leading causes of foodborne diseases worldwide. Multidrug-resistant *S. enterica* serovar Typhimurium definitive phage type (DT) 104 emerged during the early 1990s in the United Kingdom and spread worldwide thereafter (*1*). This phage-type strain harbors a chromosomally encoded genomic island, Salmonella Genomic Island 1, which is typically responsible for resistance to ampicillin, chloramphenicol,

streptomycin, sulfonamide, and tetracycline (*2*). Multilocus variable-number tandem-repeat analysis (MLVA) is an established molecular epidemiologic tool; its high-resolution power has been applied to the subtyping of a variety of bacterial species (*3*). An MLVA system has been developed for analyzing *S. enterica* serovar Typhimurium (*4*,*5*).

The design of an MLVA system relies on the analyzed genome sequences. In this study, we found and evaluated a variable-number tandem-repeat region, or locus, designated DT104o. The locus is specific to *S. enterica* ser. Typhimurium DT104, according to the sequence of NCTC 13348 (available from the Sanger Institute, http://www.sanger.ac.uk/resources/downloads/bacteria/salmonella.html). The repeat unit sequence was CTCAGAA/TTCTGAG, spanning 1952121–1952274 on the reference genome or 22 repeats of 7 nt.

We used 266 apparently independent isolates of *S. enterica* serovar Typhimurium collected during 1981–2012; 103 were from human samples and 163 from non-human sources. Bacteriophage typing was performed according to Anderson's method and scheme (*6*). Types of 100 isolates were in the DT104 group, comprising DT104, DT104B, and U302, the latter being related to DT104 (*2*); MLVA was performed by using the 5 loci (STTR3, STTR5, STTR6, STTR9, and STTR10) with slight modifications (*4*,*5*). The DT104o locus was tested by using primers o-for (5′-GTCAACATGAACTGCCCCTCA-3′), labeled with NED, and o-rev (5′-TTTGCTCTTCGCTCTTAGCAATC-3′); this spanned 1952367–1952043 on the reference sequence, resulting in a 325-bp product with 171-bp offset.

For all 266 isolates tested, the number of alleles and the Simpson's index of diversity score (*D*) identified in each locus are summarized in the Table. The 5 common and DT104o loci displayed high discriminatory power:

Table. Number of alleles and Simpson's index of diversity score in *Salmonella* enterica serovar Typhimurium definitive phage type 104 in humans*

	All, n = 266		DT104-group, n = 100	
Locus	No. alleles†	*D*	No. alleles†	*D*
STTR9	5	0.62	1	0.00
STTR5	17	0.87	10	0.76
STTR6	20	0.93	16	0.90
STTR10	25	0.90	20	0.93
STTR3	12	0.73	2	0.20
DT104o	24	0.60	23	0.92

*D, Simpson's index of diversity score.
†Including the null allele.

DT104o was specific for the DT104 group, and all 100 DT104 group isolates displayed amplified products with 13–40 repeat copy numbers; the others showed the null allele. Focusing only on the 100 DT104 group isolates, the discriminatory power of STTR9 and STTR3 were poor, whereas STTR5, STTR6, STTR10, and DT104o displayed high discriminatory powers (Table). In addition, using the 5 common loci (MLVA5) in analysis, we identified 66 types with a *D* value of 0.974; use of MLVA5 plus the DT104o locus (MLVA6) identified 83 types with a *D* value of 0.984. These results indicate that the DT104o locus is highly specific and therefore useful as an additional molecular epidemiologic marker for analyzing *S. enterica* ser. Typhimurium DT104.

Because DT104o was highly variable, 5 DT104 strains were tested for the frequency of variants at each locus after 5 serial passages by using liquid culture: cultures were diluted 1:1,000 at each passage. Sixteen colonies of each strain were tested by using MLVA6 (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/5/12-1395-Techapp1.pdf). No variants were observed in STTR3, STTR9, or STTR10. STTR5, STTR6, and DT104o each showed 1 variant of 80 colonies. The results suggest that DT104o would not be less stable than other loci.

We also found that DT104o could provide more discriminatory power to MLVA5 in some settings (online Technical Appendix Table 2). We compared 2 settings using isolates from non-human samples. Setting 1 comprised isolates 1a and 1b from an outbreak during 1996 and isolate 1c in 2007. Isolates 1a and 1b were identical by MLVA6. Isolate 1c was identical by MLVA5 but not by MLVA6. In Setting 2, three isolates obtained in different years also were identical by MLVA5, but differed from each other by MLVA6. This suggests that MLVA6 could be useful in some epidemiologic settings such as in an outbreak investigation, though more extensive study would be required to confirm this suggestion.

The DT104o locus is located at the proximal region of fragment 180 comprised of a prophage structure, which was proven to be DT104-specific in a previous study (*7*). This finding is consistent with the results of our study.

In conclusion, development of an MLVA system is dependent upon the genome sequences available, and the system is usually used for molecular subtyping of a certain serotype in a particular organism. However, a specific group of strains could cause a pandemic and become a target of public health concern, as was *S. enterica* ser. Typhimurium DT104. The MLVA system could be improved by adding loci based on the genome sequence of such pandemic strains. In this study, we showed that the newly identified DT104o locus could be useful in identification and subtyping of *S. enterica* ser. Typhimurium DT104.

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Single Genotype of *Anaplasma phagocytophilum* identified from Ticks, Camargue, France

To the Editor: Granulocytic anaplasmosis is a tickborne zoonosis caused by *Anaplasma phagocytophilum* bacteria, which are emerging in Europe. Besides infecting humans, *A. phagocytophilum* infect a wide range of wild and domestic mammals (*1*). In Europe, the *Ixodes ricinus* tick is the main vector for the bacteria, but *A. phagocytophilum* has also been detected in association with *Rhipicephalus* and *Dermacentor* spp. ticks (*2*). The climate and biotopes of the Mediterranean region are particularly favorable for several species of ticks and, therefore, for tickborne diseases.

Although *I. ricinus* ticks are rare or absent in the Mediterranean Basin, serosurveys performed on equine populations in Camargue, southern France, indicated an *A. phagocytophilum* seroprevalence of ≈10% (*3*). To investigate the prevalence and diversity of *A. phagocytophilum* bacteria in ticks in Camargue, we collected questing ticks from horse pastures and feeding ticks from horses.

Ticks feeding on horses were collected in randomly selected stables during 2007 (84 stables), 2008 (72 stable), and 2010 (19 stables). The stables were chosen among those where evidence of *A. phagocytophilum* seroconversion in horses had been previously found (*3*). In 2008 and 2010, questing ticks were collected by the dragging method in 19 pastures, around bushes, and in areas where horses spent the most time. Surveys were conducted in the spring, which represents the peak activity time of *Ixodes* ticks.

A total of 406 adult ticks were collected, representing 6 species: *Rhipicephalus bursa, R. sanguineus, R. turanicus, R. pusillus, Dermacentor marginatus*, and *Hyalomma marginatum*. Tick species were identified by morphologic criteria and molecular analyses based on mitochondrial 12S rDNA sequences (*4*). Total DNA was extracted from the ticks by using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) (*5*). *A. phagocytophilum* was detected by nested PCR targeting the 16S rDNA (online Technical Appendix 1, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp1.pdf).

Of the 406 ticks, 40 were infected with *A. phagocytophilum*. The infected group included ticks from all 6 collected species except *R. pusillus*. Infection rates among the species ranged from 0 to 22% (online Technical Appendix 2, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp2.pdf). The prevalence of *A. phagocytophilum* infection did not differ significantly between species (logistic regression model, $p = 0.76$) but was higher among questing ticks than feeding ticks ($p<0.001$; odds ratio 1.15).

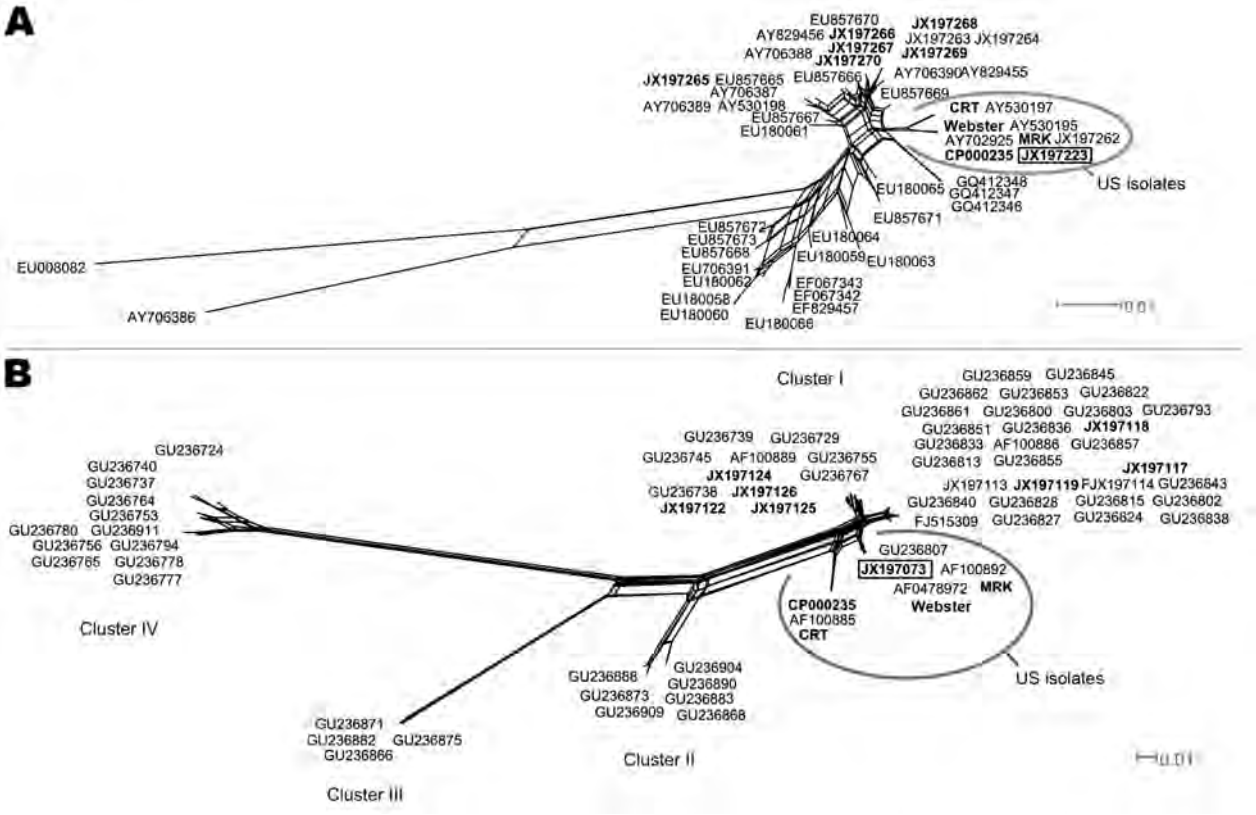

Figure. Phylogenetic networks of *Anaplasma phagocytophilum* based on *msp4* (A) and *ankA* (B) genes and built with SplitsTree4 (version 4.11.3; http://splitstree.org/) by the Neighbor-Net method. The sequences of the genotype described in Camargue, France, is framed. Sequences found in the 2 networks are in **boldface**: *A. phagocytophilum* amplified from ticks collected in Combrailles, Auvergne region, France (JX197116–JX197126 and JX197265–JX197270), a human isolate (strain Webster, EU857674 and GU236811]), an American roe deer isolate (strain CRT, JX197261 and JX197113), and *an American horse isolate (*strain MRK, AY530196 and AF153716). Scale bars indicate number of nucleotide substitutions per site.

We amplified 6 loci by nested PCR (online Technical Appendix 1) to characterize *A. phagocytophilum* genetic diversity in positive samples: *ankA*, *msp4*, *pleD*, *typA*, and intergenic regions *hemE–APH_0021* and *APH_1099–APH_1100* (National Center for Biotechnology Information annotation). The GenBank accession numbers for the nucleotide sequences are JX197073–JX197368. No polymorphism was found among the 6 loci tested in the 40 *A. phagocytophilum*–positive ticks. The genotype identified was 100% identical to the reference sequence (NC_007797) for loci *msp4*, *pleD*, and *typA* and for intergenic regions *hemE–APH_0021* and *APH_1099–APH_1100*. The *ankA* sequence was 96% similar (487 nt) to the reference sequence. The relevance of these loci as markers of diversity was verified (online Technical Appendix 3, wwwnc.cdc.gov/EID/article/19/5/12-1003-Techapp3.pdf).

To study the phylogenetic relationships between cognate sequences, we included in our analysis all sequences available in GenBank for genes *ankA* and *msp4*. To account for recombination events that affect *ankA* and *msp4* (data not shown) in phylogenetic analyses, we used Neighbor-Net networks (Figure). Phylogenetic analysis of *msp4* (Figure, panel A) indicated that the genotype of *A. phagocytophilum* from ticks in Camargue was included in a clade that also includes genotypes that infect humans and horses in the United States.

The diversity of *ankA* sequences has been described as 4 phylogenetic clusters (*6*). All sequences obtained in our study were included in cluster I, particularly in a branch composed exclusively of sequences of *A. phagocytophilum* isolated from humans in the United States (Figure, panel B).

Previous studies investigating *A. phagocytophilum* have revealed a genetic diversity that is thought to have been caused by sympatric epidemiologic cycles involving different vectors and reservoir hosts (*1*,*6*,*7*). In 5 species of ticks (40 ticks total) that we collected from a 250-km^2 area in southern France, we found only 1 genotype of *A. phagocytophilum*, which we determined to be phylogenetically close to genotypes found in the United States. Sequences phylogenetically related to bacteria in the United States were also observed in Sardinia (*8*) and Sicily (*9*).

The low diversity we found could be explained by a recent introduction of the bacteria into the area [although *A. phagocytophilum*–seropositive horses have been found in the area since 2001 (*3*)] or by a selective sweep linked to the particular ticks and host reservoir in Camargue. The 5 species of ticks that we found positive for *A. phagocytophilum* have been described as potential vectors of *A. phagocytophilum* in the Mediterranean Basin (*2*,*10*). Among the tick species in our investigation, *R. bursa* and *R. sanguineus* ticks are the 2 main carriers of *A. phagocytophilum*, and these ticks are likely to feed on humans and, thus, pose a risk of infection to the local population. Further studies are needed to address the potential effect of *A. phagocytophilum*–infected ticks on human health in this area and, more specifically, the relationship between genotype and pathogenicity.

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West Nile Virus Lineage 2 Strain in Greece, 2012

To the Editor: West Nile virus (WNV) has been in Europe at least since the 1960s (*1*). Before 2010, WNV epidemics in Europe were caused mainly by lineage 1 strains. However, in 2010, a major WNV epidemic in Central Macedonia, Greece, was caused by a lineage 2 strain (Nea Santa-Greece-2010) (*2*).

This strain also circulated during 2011 (*3*), causing a second epidemic among humans throughout the country (*4*). Although the virus was closely related to the goshawk-Hungary-2004 strain circulating in Hungary, Austria, and Italy (*5*–*7*), severe epidemics occurred only in Greece; 273 cases of West Nile neuroinvasive disease (WNND) in humans were reported during the 2 seasons (*2*,*4*).

A third epidemic occurred in 2012, and 109 WNND cases were reported (*8*). Until mid-August, most cases were in central (Attica; 29 cases) and northeastern Greece (East Macedonia and Thrace; 10 cases). In contrast, during the same period, only 3 cases were confirmed at the location of the 2010 epidemic epicenter, in Central Macedonia (*9*). This situation led to the question of whether the Nea Santa-Greece-2010 strain was responsible for the third epidemic in Greece.

In May 2012, for the second consecutive year, 12 sentinel chicken flocks (72 chickens) and 62 dry ice–baited (source of carbon dioxide) CDC mosquito traps (John W. Hock, Gainesville, FL, USA) were set in areas of Central Macedonia where WNV transmission had been high. The 3 objectives were to 1) monitor WNV activity by testing weekly for antibodies against WNV in sentinel chickens, 2) molecularly characterize the virus, and 3) assess population dynamics of the major vector species.

Serum from the sentinel chickens was tested for WNV-specific antibodies by using the ID-Screen West Nile Competition ELISA kit (IDvet, Montpellier, France). Seropositive chickens were removed from the sentinel flocks and replaced with seronegative chickens.

No enzootic activity was detected until mid-August. Antibodies were first found on August 21, in 2 chickens from the rice-growing region of western Thessaloniki (Delta and Chalkidona municipalities). The seroconversion rate in these municipalities peaked on August 27, when 4 chickens were seropositive, and remained high until September 4. Seroconversion was detected for 10 more birds in the same and other bordering municipalities, all near the rice-growing region and flood plain of the Axios, Loudias, and Aliakmonas Rivers. WNV-specific antibodies were detected in 5 more chickens sampled on September 11, in 2 chickens sampled on September 18, and in 3 chickens sampled on September 25, bringing the total number of seroconverted chickens to 26 (26.5%) of 98 (original 72 + 26 replacements). The highest rates of seroconversion among chickens were detected near rice-growing areas, where *Culex* mosquito activity (mostly *Cx. pipiens*, followed by *Cx. modestus*) was also highest. *Culex* mosquito populations peaked in mid-July, ≈30 days before the first case in a human was detected, and maintained high activity until late September.

For the 26 chickens that seroconverted, RNA was retrospectively extracted from serum by using the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Extracts were examined by using a 1-tube, real-time, reverse transcription PCR protocol and primers (WNPolUp, WNPolDo2) (3) and TaqMan probe (WNPolProb2: 5′-FAM-TCTCTCTCTTT CCCATCATGTTGT-BHQ1–3′) specific to the nonstructural (NS) 5 gene. WNV RNA was detected in 2 seropositive chickens from 2 locations, in samples taken about 1 week before seroconversion.

The positive RNA samples were reverse transcribed, and 3 overlapping fragments of the NS3 gene were amplified by PCR and then sequenced. Both complete NS3 sequences of 1,857 bp were identical (GenBank accession no. JX843471). Highest nucleotide sequence identity (99.8%) was to the strain Nea Santa-Greece-2010. Only 3 synonymous nucleotide substitutions consisting of transitions were identified, indicating minimum evolution of the virus during 2010–2012 (Figure). Molecular characterization of the 2

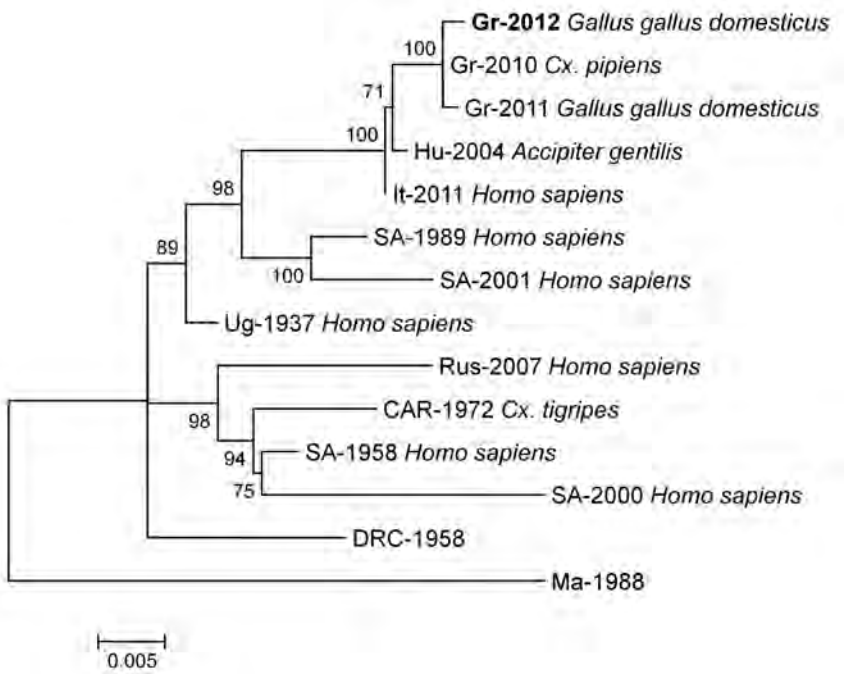

Figure. Phylogenetic tree inferred with maximum-likelihood analysis, based on complete nonstructural (NS) 3 nt sequences (1,863 bp) of lineage 2 West Nile virus strains. Isolation source is indicated in **boldface**. The general time reversible model with gamma distributed rates across sites and a fraction of sites assumed to be invariable (GTR + I + Γ) was selected as the best fitting nucleotide substitution model for the sequence dataset. The tree was mid-point rooted, and the numbers indicated on the branches are nonparametric bootstrap probabilities. Strain abbreviations indicate country, year, and GenBank accession number. Gr-2012: Greece, 2012, JX843471; Gr-2010: Greece, 2010, HQ537483; Gr-2011: Greece, 2011, JN398476; Hu-2004: Hungary, 2004, DQ116961; It-2011: Italy, 2011, JN858070; SA-1989: South Africa, 1989, EF429197; SA-2001: South Africa, 2001, EF429198; Ug-1937: Uganda, 1937, AY532665; Rus-2007: Russia, 2007, FJ425721; CAR-1972: Central African Republic, 1972, DQ318020; SA-1958a: South Africa, 1958, EF429200; SA-2000: South Africa, 2000, EF429199; DRC-1958: Democratic Republic of the Congo, 1958, HM147824; Mad-1988: Madagascar, 1988, HM147823. Scale bar indicates nucleotide substitutions per position. *Cx., Culex*.

isolates actively circulating where cases in humans had been confirmed suggests that the strain responsible for the 2012 epidemic in Greece was again Nea Santa-Greece-2010.

The surveillance system successfully identified areas with increased levels of vector and WNV activity. This information was quickly disseminated to public health authorities so they could intensify control measures in the affected areas. After the first seroconversions in chickens were detected, 9 new WNND cases and 2 cases without central nervous system manifestations in humans were reported from residences near (6–15 km) the chicken coops that housed the seropositive birds (*8*). Of the 9 cases of WNND, 7 were reported after the rate of chicken seroconversion peaked.

The continuous occurrence of WNV epidemics in Greece indicates that the virus will probably remain a serious threat. This probability is further supported by the epidemic pattern in the United States, where ≈10 years after its introduction WNV is still causing large epidemics (*10*). Surveillance programs that can accurately determine public health risk and lead to timely vector control interventions are needed to prevent human infection.

Particularly in areas such as Europe, where numerous strains of different virulence coexist, molecular identification of the circulating viruses is necessary for risk assessment. Captive sentinel chicken surveillance with repetitive sampling might be an informative tool.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Reindeer Warble Fly–associated Human Myiasis, Scandinavia

To the Editor: We report migratory myiasis that occurred during 1991–2012 caused by the reindeer warble fly, *Hypoderma tarandi* (online Technical Appendix Figures 1, 2, wwwnc.cdc.gov/EID/article/19/5/13-0145-Techapp1.pdf), in 7 tourists to reindeer habitats of northern Scandinavia. We also report 2 additional women (patients 8 and 9), independent of each other, who were asymptomatic but sought medical care in August 2012 after finding 30–60 parasite eggs in scalp hair 3 days after hiking in Kebnekaise and Jämtland Mountains (northern Sweden), respectively.

Patients 1–7 (Table) had enlarged regional lymph nodes and migratory dermal swelling of the head and upper face. Rounded cutaneous swelling of 2–5 cm occurred 1 at a time, persisted for 1–3 days, and reappeared after 2–34 days.

In mid-January 2009, 4 months after initial symptoms, patient 1 felt a sudden pain in his left eye; 10 days later, an ophthalmologist discovered an intraocular larva (online Technical Appendix Figure 3). Patient 3 had a swelling on his forehead, which reappeared 2′ before his right eyelid swelled; the day after the eyelid swelling disappeared, vision decreased in his right eye. Patients 1, 3, and 7 underwent eye surgery; 1 living larva was extracted from each patient. Patients 3 and 7 lost vision in the affected eye.

For 5 patients, ivermectin was administered orally (≈200–350 μg/kg body weight), on 3–5 occasions in relation to the swellings. Patients 8 and 9 also each received 1 dose of ivermectin; they remained asymptomatic. Patient 3 received the first dose on day 5 after the living larva was extracted because of a new swelling. Swelling recurred on 3 occasions 2 weeks–1.5 months after surgery. In patient 7, swelling reappeared on several occasion 10–30 days after eye surgery, indicating that retrieval of 1 larva does not exclude concomitant occult infestations. This probably was also the case for patient 2, who had a swelling on his upper forehead when pain developed at the root of his nose, where a new swelling appeared 4 days later.

The 3 larvae removed from patients 1, 3, and 7 were identified as *H. tarandi*, 2 by morphology and 1 by molecular-specific amplification and sequencing (*1*). Antibodies against hypodermin C, an enzyme released by the larva during migration in host tissues, were detected in 5 of the symptomatic patients (*2*,*3*).

H. tarandi eggs take 4–7 days to hatch, depending on the temperature of the hair layer (*4*); thus, patients 8 and 9 were treated soon after oviposition and were seronegative. Newly hatched *H. tarandi* larvae can easily dry, so their chance of survival is higher when they are close to scalp skin. Eggs from patient 2 were initially misidentified as head lice eggs but were eventually identified as *H. tarandi* by T.G. Jaenson (Uppsala). Published photographs of the *H. tarandi* eggs alongside the eggs of head lice (*5*) helped identify *H. tarandi* eggs in patients 8 and 9. According to those patients, *H. tarandi* eggs could not be removed from the hair with a lice comb. The *H. tarandi* fly is well adapted to sub-Arctic climate; nearly all reindeer were found to be infested in some districts of northern Finland and Norway (*6*). Reindeer habitats attract tourists, mostly during summer. *H. tarandi* is mainly active on warm summer days; warm weather perhaps does not encourage persons to cover their heads, which may predispose for oviposition. Also, persons moving around probably attract more flies than do those staying still, and strong wind, rain, and temperatures <10°C–12°C are thought to inhibit the warble fly's flight activity and oviposition (*7*).

Awareness of human infestation by *H. tarandi* warble flies increased in Sweden and Norway after news media in Sweden described patient 2 (*5*; www.lakartidningen.se/engine.php?articleId=14643). This publication helped in the recognition of symptoms and in shortening diagnostic delay in patients 3–6, 8, and 9. Of the 3 cases for which diagnosis was not delayed, patients 4 and 5 were children of a physician who read our publication and recognized the symptoms; patient 6, herself a physician, also read the article (*5*). Increased awareness, rather than increased incidence, explains the emergence of new cases. Nine of 12 cases of proven *H. tarandi* myiasis found in the literature occurred in persons who had ophthalmomyiasis interna (*3*,*8*,*9*); migratory dermal swellings, the clinical signature of hypodermosis, have been reported only in 1 case (*10*). Such swellings occurred in all the patients reported here, suggesting that clinicians overlooked this finding, possibly because of the overtaking severity of eye complications and the reporting of most previous cases by ophthalmologists (*3*,*8*,*9*). Persons who seek care for migratory dermal swellings during August–December should be asked about recent travel to reindeer habitats.

For 3 patients with ophthalmomyiasis reported here, ophthalmologists initially had difficulty establishing a diagnosis, raising the possibility that some cases of "idiopathic" uveitis from *H. tarandi*–endemic areas may be caused by *H. tarandi*. Ophthalmomyiasis should be considered in cases of unilateral uveitis, lens subluxation, and suspicion of intraocular foreign body (*3*,*8*,*9*). Eosinophilia might be absent and should not be used to guide treatment.

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Table. Myiasiss caused by warble reindeer fly (*Hypoderma tarandi*), Scandinavia, 1991–2012*

Characteristic	Case-patient no. 1	2	3	4†	5	6	7‡
Age, y/sex	8/M	10/M	10/M	10/M	6.5/F	56/F	28/M
Date of symptom onset	2008 Sep 15	2009 Aug 15	2010 Sep 12	2010 Sep 3	2010 Dec 3	2011 Oct 10	1991 Aug
Signs and symptoms	Enlarged occipital and retroauricular lymph nodes	Eggs in scalp hair; enlarged occipital and cervical lymph nodes	Forehead swelling	Enlarged occipital and cervical lymph nodes	Forehead and eyebrow swelling	Occipital swelling	Occipital swelling
Travel							
Dates	2008 late Jul–early Aug	2009 Jul 20–Aug 7	2010 Jul 7–16	2010 Aug 14–19		2011 Jul	1991 Jul
Destination	Norway, extreme northeast: patient resides in Troms County where reindeer are occasionally seen	Sweden: Lapland	Sweden: Kiruna, Riksgränsen, Abisko (several short hiking tours) Norway: Bodö	Sweden: family undertook a 5-d hiking tour in Lunndörrsfjällen, a mountain area in Jämtland County		Norway: Lapland	Sweden: short trips to Arvidsjaur (Lapland) and Jämtland Mountains
Observed reindeer	Yes	Yes§	No	Yes; at close range		Yes	Yes
Migratory swellings, no.	Temple, 1	Head, 1; forehead, 5	Forehead, 4; eyelid, 1; behind ear, 1	Forehead and eyelid, 5	Forehead and eyelid, 2	Head, temple, eyelid, >5	Head, >5
Fever	Yes	No	No	Yes	No	No	No
Eosinophilia (highest value)¶	Yes (1,0)	Yes (0,6)	Yes (0,8)	Yes (3,8)	No	No	Unknown
Other signs and symptoms	Uveitis, failure to gain weight	Localized exanthema	Itching of scalp, enlarged retroauricular lymph nodes, headache, uveitis	Fever, headache, nausea	No	Enlarged nuchal lymph nodes	Uveitis, glaucoma, retinal hemorrhage
Diagnostic delay, d#	74	25	14	0	0	1	>60
Diagnosis	Positive serology, morphologic identification of larva	Positive serology, identification of eggs	Positive serology, molecular identification of larva	Positive serology	Positive serology	Negative serology**	Negative. serology in 2011 and 2012, morphologic identification of larva in 1991
Drugs received							
Ivermectin	No	5 doses	5 doses††	5 doses	3 doses	2 doses	No
Antihistamines	No	Yes	Yes	Yes	Yes	Yes	Unknown
Oral steroids	Yes	No	No	Yes	Yes	Yes	Steroids, antimicrobial drugs given after surgery
Outcome	After eye surgery glaucoma; visual acuity 0,9	Good	Eye surgery; visual loss, right eye	Good	Good	Good	Eye surgery; visual loss, right eye

*Patients 8 and 9 are not included in the table because myiasis did not develop in them.
†Patients 4 and 5 are siblings.
‡Patient 7 was discovered by the father of patients 4 and 5 among his acquaintances, suggesting the possibility of additional unreported cases in the population.
§In Jukkasjärvi (Sweden), the child had visited an enclosure where the reindeer were agitated because of swarms of flies.
¶Referent 0–0.5 × 10^9/L.
#Interval between date of first visit for myiasis-associated symptoms and date when treatment began.
**The diagnosis could not be confirmed, but her clinical picture and response to treatment were similar to those of other patients.
††Ivermectin was given first after eye surgery.

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Azole-Resistant *Aspergillus fumigatus*, Iran

To the Editor: *Aspergillus fumigatus* causes a variety of diseases in humans. The drugs recommended for treatment of *Aspergillus* diseases are the mold-active azole antifungal drugs (*1*). However, a wide range of mutations in *A. fumigatus* confer azole resistance, which commonly involves modifications in the *cyp51A* gene (*2*), the target for azole antifungal drugs.

Azole resistance is thought to be selected for as a result of patient therapy or exposure to azole compounds in the environment; resistance in clinical *A. fumigatus* isolates has been increasingly reported in several European countries, Asia, and the United States (*3*–*7*). The most frequently reported resistance mechanism is a 34-bp tandem repeat (TR_{34}) in combination with a substitution at codon 98 (TR_{34}/L98H) (*4*); this mechanism is believed to have been selected for through environmental exposure to azole fungicides.

Because routine in vitro susceptibility testing of clinical *Aspergillus* isolates is not common in many centers worldwide, the prevalence of azole resistance might be underestimated. We investigated the prevalence of azole resistance in clinical *A. fumigatus* isolates stored for 6 years (2003–2009) at Tehran University Mycology Reference Centre and Islamic Azad University, Ardabil Branch, Iran.

We investigated 124 clinical *A. fumigatus* isolates obtained from patients with *Aspergillus* diseases (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/5/13-0075-Techapp1.pdf). We conducted strain identification, in vitro antifungal susceptibility testing, and sequence-based analysis of the *Cyp51A* gene, as described (*4*). We performed microsatellite genotyping of all *A. fumigatus* isolates for which the MIC of itraconazole was ≥16 mg/L (*8*) by using a short tandem repeat *A. fumigatus* assay, and we compared the results with those reported for the Netherlands (20 isolates) and other European countries (24 isolates) (online Technical Appendix Figure).

The distribution of azole-resistant and wild-type *A. fumigatus* isolates examined in this study, according to year of isolation, is shown in online Technical Appendix Table 1. Of 124 *A. fumigatus* isolates, 4 grew on the wells containing itraconazole and voriconazole, indicating a multidrug-resistant phenotype. Of these resistant isolates, 3 were from patients with chronic pulmonary aspergillosis and 1 was from a patient with allergic bronchopulmonary aspergillosis (Table).

Sequence analysis of the *CYP51A* gene indicated the presence of TR_{34}/L98H in 3 isolates and no mutations in the other isolate (Table). The first TR_{34}/L98H isolate had been recovered in 2005, which is relatively early compared with reported isolations in other countries (online Technical Appendix Table 2). Microsatellite typing of 6 short tandem repeat loci demonstrated identical patterns for 2 of the 3 azole-resistant isolates from Iran, but the TR_{34}/L98H isolates from Iran did not cluster with those from the Netherlands and other European countries, indicating no close genetic relatedness (online Technical Appendix Figure).

The TR_{34}/L98H azole resistance mechanism was first described in 1998 in the Netherlands; since then, its presence in clinical and environmental *A. fumigatus* isolates in multiple European countries and recently in Asia has been increasingly reported (online Technical Appendix Table 2) (*3*–*7*). In the study reported here, prevalence of azole resistance in clinical *A. fumigatus* isolates obtained from patients in Iran was 3.2%; most isolates exhibited the TR_{34}/L98H resistance mechanism. The fact that the first TR_{34}/L98H isolate was found relatively early, in 2005, underscores the possibility that prevalence

Table. Characteristics of 4 azole-resistant clinical *Aspergillus fumigatus* isolates, Iran*

Isolate	Underlying disease	Previous azole exposure	34-bp tandem repeat†	Amino acid substitution in *cyp*51A gene‡	MIC, mg/L			
					Amphotericin B	Itraconazole	Voriconazole	Posaconazole
T-IR-AF 12	CPA	Yes	Positive	L98H	0.5	≥16	4.0	0.5
T-IR-AF 17	CPA	No	Positive	L98H	0.5	≥16	4.0	0.5
T-IR-AF 433	CPA	Yes	Negative	ND	0.5	≥16	8.0	0.5
T-IR-AF 890	ABPA	No	Positive	L98H	0.5	≥16	8.0	0.25

*CPA, chronic pulmonary aspergillosis; ND, not detected; ABPA, allergic bronchopulmonary aspergillosis.
†34-bp tandem repeat in the promoter region of *CYP51A* gene.
‡The numbers indicate the position at which an amino acid change occurs. Nucleotides are numbered from the translation start codon ATG of *cyp51A*.

of azole resistance might be underestimated in many countries because in vitro susceptibility testing of *A. fumigatus* is not routinely performed.

Microsatellite genotypic analysis of *A. fumigatus* isolates from the Netherlands and various European countries showed that the genetic diversity of TR_{34}/L98H isolates is lower than that of wild-type controls (*8*). It has been suggested that TR_{34}/L98H isolates might have a common ancestor that developed locally and subsequently migrated across Europe. In contrast, genotyping of TR_{34}/L98H originating from India suggested a different dynamic; all environmental and clinical TR_{34}/L98H isolates from India shared the same multilocus microsatellite genotype not found in any other analyzed samples, from within India or from the Netherlands, France, Germany, or the People's Republic of China (*9*). The molecular epidemiology of the TR_{34}/L98H isolates from Iran suggests that they cluster apart from the European isolates, indicating that migration from Europe to Iran, or vice versa, is unlikely. Genotyping of more TR_{34}/L98H isolates from the Middle East and comparison with those from India would enhance understanding of the origin and geographic spread of TR_{34}/L98H.

Our study indicates that TR_{34}/L98H was in Iran in 2005; this finding adds to the growing list of regions where acquired resistance in *A. fumigatus* of environmental origin is documented. From a global perspective, fungicide use is second highest in the Asia–Pacific regions (24%), preceded only by western Europe (37%) (*10*). For a bettering understand of the scale of this emerging public health problem and for insight into the dynamics of geographic migration, surveys of fungal culture collections for TR_{34}/L98H and molecular typing studies are warranted. These data would be useful not only for clinical management of *Aspergillus* diseases but also for enabling policy makers to develop strategies that prevent resistance selection by the environmental route.

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Search for Possible Additional Reservoirs for Human Q Fever, the Netherlands

To the Editor: Q fever is a zoonosis caused by the bacterium *Coxiella burnetii*. The Q fever outbreak in the Netherlands affected ≈4,000 humans during 2007–2010 (*1*). In this outbreak, 1 genotype of *C. burnetii* appeared to be responsible for abortions in small ruminants and for clinical disease in humans (*2*,*3*). However, little is known about the outbreak genotype and the prevalence of *C. burnetii* in possible additional reservoirs for human Q fever (i.e., cats, dogs, horses, sheep, and cattle) in the Netherlands.

We aimed to search for possible additional reservoirs for human Q fever in the Netherlands. Placentas from 15 cats, 54 dogs, and 31 horses were collected in 2011 at 5 veterinary practices. Placentas were collected by targeted sampling at breeding facilities and during parturition with veterinary assistance. In addition, 27 ovine, 11 caprine, 16 porcine, 8 equine, and 139 bovine placentas (originating from aborting animals from throughout the Netherlands that were submitted in 2011 to investigate the abortion cause) were included in the study. Samples were stored at –20°C before testing.

DNA was extracted from the allantochorion of the placenta and analyzed as described (*2*). Samples with sufficient DNA load (cycle threshold [C_t] value <32) were typed by using 2 multilocus variable-number tandem-repeat analyses (MLVA) genotyping methods (MLVA-12 and MLVA-6), and the multispacer sequence typing method (*3*–*5*). Two *C. burnetii* strains from the Netherlands representing the outbreak genotype (X09003262, 3345937) and the Nine Mile RSA 493 were included as reference. For prevalence calculations, the Netherlands was divided in a southern part, comprising the Q fever hot spot area of notified cases in humans and small ruminants during the 2007–2010 epidemic (*1*,*6*), and a northern part, comprising the rest of the country.

C. burnetii DNA was not detected in placentas from cats, goats, or pigs. *C. burnetii* DNA was detected in 4 (7% [95% CI 0.4–14.4]) of 54 canine placentas; 3 from the north and 1 from the south of the Netherlands. *C. burnetii* DNA was detected in 3 (8% [95% CI 0.0–16.1]) of 39 equine placentas, all from the north of the country, without known abortion history. *C. burnetii* DNA was detected in 7 (26% [95% CI 9.4–42.5]) of 27 ovine and in 33 (24% [95% CI 16.7–30.8]) of 139 bovine placentas. The prevalence of *C. burnetii* DNA–positive ovine and bovine placentas from the north and the south did not differ significantly.

The *C. burnetii* DNA load in the placentas from dogs (C_t value 37.4–38.0) and horses (C_t value 35.4–37.4) was too low to be suitable for genotyping. Typing of 1 positive sheep sample resulted in an incomplete genotype, which is related to the outbreak genotype (sheep 192, Figure). Seven of the 33 *C. burnetii* DNA–positive bovine placentas were suitable for typing. One sample had a genotype similar to the outbreak genotype (*2*,*3*). Six other samples revealed a (partial) genotype related to bovine genotypes from the Netherlands (*2*,*5*,*7*), including a novel one. MLVA-6 and multispacer sequence typing results were consistent with the MLVA-12 results (Figure).

Results give no indication for major reservoirs of *C. burnetii* in cats, goats, and pigs in the Netherlands in 2011. However, the low numbers of placentas may have biased the results. Dogs and horses should be considered as reservoirs for *C. burnetii*. The detection of *C. burnetii* DNA–positive placentas in dogs and horses in the northern part of the country indicates the presence of a true reservoir rather than a spillover effect from the contaminated environment in the south. This observation is consistent with a reported seroprevalence of 13% in dogs in the Netherlands in 1992 (*1*). Until now, horses had been discussed as a risk factor in the Q fever outbreak in the Netherlands (*8*).

Prevalence data from sheep and cattle suggest that *C. burnetii* is present in placentas in 25% of the abortion cases in these species. Presence of the outbreak genotype of *C. burnetii* in sheep has been observed (*2*,*5*), indicating sheep are a reservoir for Q fe-

ver in humans. Genotyping data show a distinct genotype in 6 of the 7 cattle samples in accordance with previous work (*2*,*5*,*7*). However, the outbreak genotype was detected in 1 sample from a cow. Whether this is an incidental finding or the first observation of the outbreak genotype being transferred to the cattle population is not clear. If the latter, exposure to cattle also possibly might become a risk factor for human Q fever, in addition to goats and sheep.

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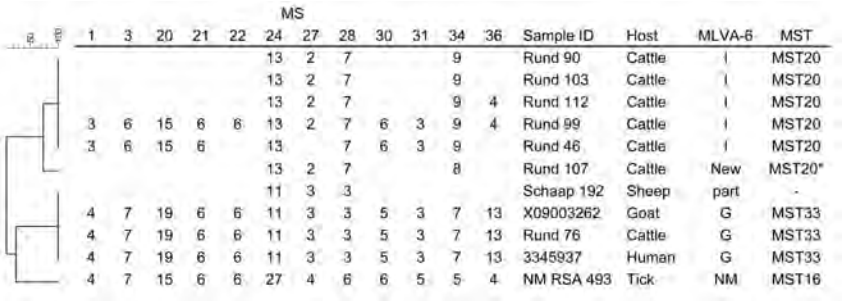

Figure. Phylogenetic tree of the genotypes of *Coxiella burnetii* from the samples of this study based on multilocus variable-number tandem-repeat analyses (MLVA) including 12 loci (MLVA-12). Repeats per locus are shown, and open spots indicate missing values. MLVA-6 are results of the analysis with 6 MLVA loci (*3*). MST are results of the analysis with multispacer sequence typing (MST) (*5*). MLVA 6 loca (MLVA-6) and MST revealed full genotypes unless stated otherwise. Two strains representing the outbreak genotype of *C. burnetii* (X09003262, 3345937) in the Netherlands and the Nine Mile (NM) RSA 493 are included as reference. MS, mini satellite; G and I, MLVA-6 genotypes of *C. burnetii* as published (*3,7*); MSTxx, MST genotypes as published (*5*). *Based on partial genotype; part, partial genotype. – (in MST column) indicates no results obtained. Scale bar indicates percentage similarity.

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Scalp Eschar and Neck Lymphadenopathy Caused by *Rickettsia massiliae*

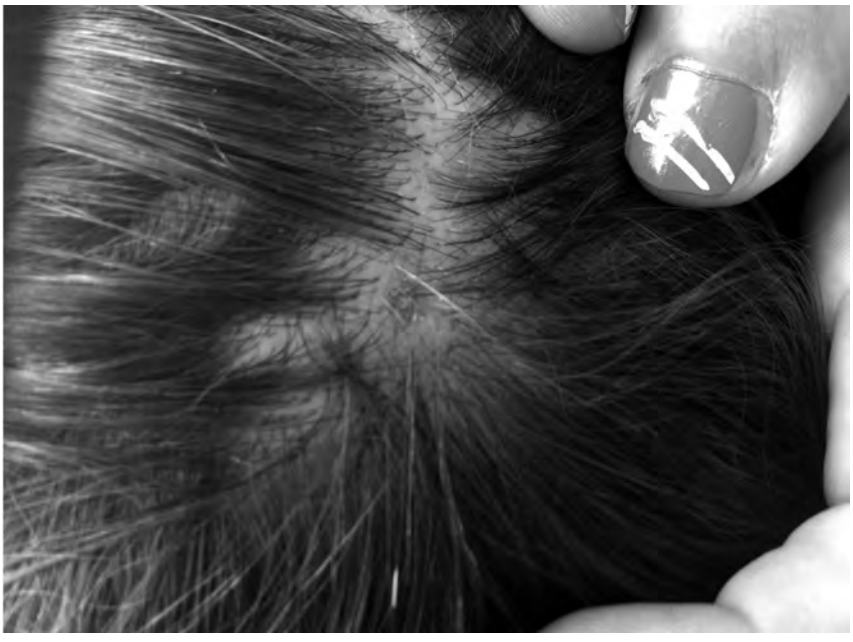

Figure. Residual alopecia 10 weeks after tick bite in 13-year-old boy with scalp eschar and neck lymphadenopathy caused by *Rickettsia massiliae*. Printed with permission from N.C. (photographer and author) and from parents of the patient.

To the Editor: Scalp eschar and neck lymphadenopathy is a common clinical entity that most frequently affects women and children during spring and fall. It is usually caused by *Rickettsia slovaca* and *R. raoultii*. Typical clinical signs are a scalp lesion at the tick bite site and regional, often painful, lymphadenopathy. Acute disease can be followed by residual alopecia at the bite site (*1*,*2*). Two designations have been proposed for this syndrome: tick-borne lymphadenopathy and *Dermacentor*-borne necrosis-erythema-lymphadenopathy (both have been associated with *R. slovaca*); however, the most generic and all-inclusive term is scalp eschar and neck lymphadenopathy.

R. massiliae belongs to the spotted fever group rickettsiae, is distributed worldwide, and is transmitted by ticks of the genus *Rhipicephalus* (*3*). To our knowledge, only 3 cases of *R. massiliae* infection in humans have been documented and confirmed by molecular methods. The first case was detected in a blood sample from a patient in Italy who had Mediterranean spotted fever (*4*); the second case was in a patient in southern France who had spotted fever and acute loss of vision (*5*); and the third case was in a woman in Argentina who had fever, a palpable purpuric rash, and tache noire (*3*). We report a case of *R. massiliae* infection that resulted in scalp eschar and neck lymphadenopathy.

On May, 10, 2012, a 13-year-old boy was examined for headache, high fever, and right painful neck and occipital swelling. Six days earlier, a tick had been removed from the top of his scalp, after which signs and symptoms arose and gradually worsened.

Physical examination revealed temperature 39.5°C, pulse rate 70 beats/min, and respiratory rate 20 breaths/min. The boy appeared to be in good condition. An ≈1-cm black eschar was noted at the site of the tick bite. Palpation of the neck revealed painful bilateral adenopathies. Other lymph nodes in the occipital region were enlarged. No exanthema was noted, the liver was palpable 1 cm under the costal margins, and the spleen was not enlarged. Laboratory evaluation indicated blood cell counts and liver and kidney function within reference limits, mild elevation of inflammatory markers (C-reactive protein 1.2 mg/dL [reference <0.5 mg/dL]), and elevated erythrocyte sedimentation rate (43 mm/h). Ultrasonography of the neck confirmed the presence of numerous, enlarged, oval lymph nodes (maximum 17 mm) with hilar vascularity within normal limits. A scalp eschar biopsy sample and acute- and convalescent-phase (day 30) serum samples were sent to the Istituto Zooprofilattico Sperimentale della Sicilia.

The patient was given doxycycline at 100 mg 2 times per day. Signs and symptoms began to improve 48–72 h later and gradually disappeared. Fever was gone after 3 days, and the other symptoms had regressed after 7 days.

Serologic testing for *R. conorii* was performed by microimmunofluorescence with the *R. conorii/R. typhi* IgG MIF Kit (Fuller Laboratories, Fullerton, CA, USA). Total DNA was extracted from blood and the eschar by GenElute Mammalian Genomic DNA Miniprep (Sigma-Aldrich, St. Louis, MO, USA). To detect *Rickettsia* spp. DNA, we tested nucleic acids by PCR with a set of primers that amplify a 256-bp region of the gene encoding the 17-kDa antigen (*6*). To obtain information about *Rickettsia* spp., we amplified regions of the genes *gltA* (*7*,*8*), *ompA* (*7*), and *ompB* (*9*). PCR products were purified by the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA), quantified, and sent for sequencing to Macrogen Inc. (Amsterdam, the Netherlands).

Obtained sequences were aligned and analyzed by using Bioedit software (Ibis Biosciences, Carlsbad, CA, USA) and ClustalW version 2.0.10 (www.ebi.ac.uk/clustalw). DAMBE (http://dambe.bio.uottawa.ca/dambe.asp) and MEGA (www.megasoftware.net) software were used to obtain similarity percentages among analyzed sequences. To characterize Rickettsia spp., we used nucleotide sequence identity to reference strains (*10*).

Convalescent-phase serum was positive for *R. conorii*; IgG titer was 64. Sequence analysis of purified PCR products obtained from the eschar identified the isolate as *R. massiliae*. With respect to the reference strain *R. massiliae*, pairwise nucleotide sequence identity was 99% for the *gltA* gene (GenBank accession no. JN043507), 99% for the *ompA* gene (accession no. JQ480842), and 97% for the *ompB* gene (accession no. AF123714). Phylogenetic analysis (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1169-Techapp1.pdf) also confirmed the identity of the *Rickettsia* species.

Considering the diagnosis of *R. massiliae* infection and the patient who had acute vision loss (*5*), this patient was called back for a fundus examination, which showed no changes. At the time of this visit, a small area of alopecia at the eschar site was observed (Figure). Unfortunately, the tick had been discarded and was not available for genus and species identification.

The presence of *R. massiliae* in Italy demonstrates that this Rickettsia species can cause scalp eschar and neck lymphadenopathy. Further studies are needed to complete the list of microorganisms that can cause this condition and to understand if they can be associated with minor findings (e.g., alopecia, painful eschar, high fever).

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Mycobacterium tuberculosis Complex in Remains of 18th–19th Century Slaves, Brazil

To the Editor: Nineteenth century Rio de Janeiro, Brazil, was marked by increased illness and deaths from tuberculosis (TB). By the twentieth century, it was still believed that most TB cases in the Americas originated from Europe; the "virgin soil" hypothesis for African (*1*) and Amerindian populations was accepted. However, modern and archeological DNA evidence confirms the wide distribution of *Mycobacterium tuberculosis* complex (MTC) and TB in the Old and New Worlds.

Rio de Janeiro was a main entry port for millions of Africans captured for the slave trade. Pretos Novos (New Blacks) Cemetery (PNC; 1769–1830) was created in Rio de Janeiro as a burial ground for the many slaves who died at market. Comingled bone

fragments (≈5,000) from ≈30 persons were recovered at PNC; most bones were broken and had been exposed to fire (*2*,*3*). Bioanthropological analysis determined most of the bones were from men 18–25 years of age (*2*); none had lesions consistent with TB.

Femurs from 16 persons were surveyed for *M. tuberculosis* ancient DNA (aDNA). The thick shafts of femur offered a preserved condition for molecular analysis, and the bones could be individualized, avoiding duplication of samples. Paleogenetic investigation guidelines were followed. Sample preparation, aDNA extraction, and PCR were performed at the Paleogenetics Unit (Oswaldo Cruz Foundation, Rio de Janeiro) an isolated environment exclusively dedicated to aDNA research.

Before removal of the bone surface, samples were decontaminated by ultraviolet light (15 min/all sides), frozen in liquid nitrogen, and subjected to manual trituration. Bone powder (≈200 mg) was then incubated with digestion buffer (56°C, 48–72 h) as described (*4*). aDNA hybridizations with MTC probes were conducted as described (*4*). By using 2 segments of mitochondrial DNA (mtDNA) hypervariable segment I (HVS-I) target, we determined the ancestry of the humans from whom the bones were derived (*4*). To control for recent contamination, we compared the HVS-I sequences with those in GenBank and also in a database for the laboratory staff.

Using the hybridization assay with insertion sequence (IS) 6110 target, we detected MTC aDNA in bones from 4/16 persons (samples PN1, PN8, PN13, PN15); 3 of these samples (PN1, PN8, PN13) were also positive for IS1081 target, confirming MTC infection (Table). HVS-I target was retrieved from 3 samples (PN6, PN14, PN15), which enabled determination of the human mtDNA haplotypes (L3e2, L3d1, L1c2, respectively) (Table). The haplotypes showed that the 3 persons were of African descent (GenBank accession nos. JQ639893–Q639895). Our findings are consistent with those from studies based on current African populations, which show that haplotype L1c is restricted to central Africa (*5*) and L3d and L3e are most frequently found in western and central Africa, respectively (*6*).

Historic data (*3*) showed that 95% of persons buried in PNC were New Blacks, meaning they were born in Africa and died just after arriving in America. Our mtDNA results confirm historic and genetic records that indicate a large percentage of persons brought to Brazil as slaves originated from western–central and western Africa. This makes the PNC samples unique for the paleogenetic purpose of this investigation.

The endemicity of TB in Rio de Janeiro during the colonial period was confirmed by Jaeger and colleagues, who demonstrated MTC infection in 56.6% of persons with European ancestry buried at Nossa Senhora do Carmo Church (*4*). The difference in the frequency of MTC found in the remains of slaves buried in PNC and of Europeans buried at Nossa Senhora do Carmo Church may be explained by the types of samples analyzed and the epidemiologic conditions of both groups. The cremation of corpses at PNC may also partly explain the difference. The finding of MTC aDNA in the remains of 25% of persons buried at PNC could be an underestimation of infection.

Our finding that some of the slaves buried in PNC had TB infection

Table. Results of genetic analyses of *Mycobacterium tuberculosis* complex hybridization and human mtDNA haplotypes from human bone samples collected from Pretos Novos Cemetery, Rio de Janeiro, Brazil*

	TB hybridization		Nucleotide position of mtDNA hypervariable segment I†												mtDNA
Sample	IS6110	IS1081	129	145	148	172	187	189	213	223	278	311	319	320	haplotype
CRS	NA	NA	G	G	C	T	C	C	G	C	C	T	G	C	H
PN1	+	+													
PN2	–	–													
PN3	–	–													
PN4	–	–													
PN5	–	–													
PN6	–	–	G	G	C	T	C	T	G	T	C	C	G	T	L3e2
PN7	–	–													
PN8	+	+													
PN9	–	–													
PN10	–	–													
PN11	–	–													
PN12	–	–													
PN13	+	+													
PN14	–	–	G	G	C	T	C	T	G	T	C	T	A	C	L3d1
PN15	+	–	A	A	C	T	T	C	A	T					L1c2
PN16	–	–													

*The cemetery was used as a burial ground for African slaves who died in slave markets during 1769–1830. Blank cells mean target could not be retrieved. mtDNA, mitochondrial DNA; TB, tuberculosis; IS, insertion sequence; CRS, Cambridge Reference Sequence (accession no. AB055387); NA, not applicable; PN, bone samples from humans buried in Pretos Novos Cemetery; Abs, absence of nucleotide.
†The prefix 16 has been omitted from the nucleotide numbers.

when they arrived in Brazil is in agreement with previous findings of the differential distribution of TB and with a tuberculin survey on the African continent, supporting the hypothesis of native African TB (*7*,*8*). Therefore, the hypothesis of Africa as virgin soil for TB (*1*,*9*) cannot be easily supported. The incidence of TB among the slaves/Blacks in Rio de Janeiro was less than expected given their social and sanitary conditions (*10*), especially in a TB-endemic situation (*4*). Previous exposure to MTC might explain their apparent relative resistance.

Other evidence showing African contact with Europeans before the sixteenth century, supports the existence of TB in Africa (*8*), and TB was prevalent in urbanized centers along coastal areas of western Africa (*7*,*8*). Although some of those cases were probably the result of European contact, it is not possible to exclude that some were caused by TB native to Africa. We can affirm that persons buried in PNC, who were transported to Brazil as slaves from Africa, brought TB infection with them; whether the infection was caused by European TB endemic to Africa or by TB native to Africa is not known.

Acknowledgments

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Treatment of Listeriosis in First Trimester of Pregnancy

To the Editor: Foodborne infections with *Listeria monocytogenes* continue to be dangerous and disruptive. A 2011 outbreak in the United States, linked to cantaloupes, affected 147 persons; 33 persons died, and 1 pregnant woman experienced a miscarriage (*1*). Moreover, the incidence of listeriosis has been rising in several European countries (*2*). Compared with the general population, pregnant women are at markedly increased risk of acquiring listeriosis (*3*). Women who are infected with *L. monocytogenes* in the third trimester of pregnancy are typically treated with antimicrobial drugs until the child's delivery (*3*). However, the optimal treatment regimen for listeriosis early in pregnancy is unknown.

We cared for a 28-year-old, previously healthy woman who sought treatment at 12 weeks' gestational age with fever, headache, and neck stiffness; blood cultures were positive for *L. monocytogenes*. Lumbar puncture on admission to our hospital in Boston, Massachusetts, in December 2011, revealed clear fluid and an opening pressure of 15 mm Hg; 1 leukocyte was observed per high-powered field, and cultures of the cerebrospinal fluid were sterile. Pelvic ultrasound showed no abnormalities of the fetus, gestational sac, or uterus.

We treated the patient's condition with intravenous ampicillin

for 2 weeks, 2 g every 4 hours, and gentamicin, 100 mg every 8 hours, followed by ampicillin alone for 2 weeks. Shortly after the antimicrobial drugs were initiated, the patient defervesced and her blood cultures cleared. Her hospital course was complicated by spinal headache and transient acetaminophen-induced liver injury, but she was eventually discharged to her home in good condition. Blood cultures taken after discontinuation of antimicrobial agents were sterile, and the remainder of her pregnancy was unremarkable.

She ultimately gave birth to a healthy 2,405-g boy with Apgar scores of 4 and 7 (at 1 and 5 min, respectively) at 35.1 weeks' gestation by spontaneous vaginal delivery. Pathologic examination of the placenta showed no evidence of chorioamnionitis, villitis, or parenchymal abscesses, and placental cultures were sterile. The patient and her child are currently doing well without obvious sequelae of infection.

Listeriosis in early pregnancy presents a unique challenge for the infectious diseases clinician. Up to 30% of *L. monocytogenes* infections in pregnancy result in stillbirth, miscarriage, or preterm labor, and approximately two thirds of surviving neonates are infected (*4*). *L. monocytogenes* uses 2 surface proteins, InlA and InlB, to invade host cells, including the placenta (*5*). Once established within the placenta, *L. monocytogenes* forms microabscesses, which can lead to recurrence of infection (*6*). A recent study in which researchers used a guinea pig model suggests that eradication of microabscesses from the placenta may be critical to achieving the cure of the mother and the prevention of fetal illness and death (*7*).

What, then, is the optimal treatment strategy to cure the mother and sterilize the placenta? In a large case series of pregnant women with listeriosis, most patients were given a b-lactam antimicrobial drug, with or without gentamicin (*6*). However, most women in this case series were in their third trimester of pregnancy and received treatment until delivery. In women who are infected in the first or second trimester, continuing intravenous antimicrobial drugs until delivery is impractical, and the efficacy of oral antimicrobial agents in preventing recurrence of infection is unknown.

Our case demonstrates that 4 weeks of intravenous therapy can sterilize the placenta and enable good maternal and fetal outcomes in a woman infected with listeriosis in the first trimester. We also identified 13 case reports of women in whom listeriosis developed in the first or second trimester of pregnancy (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/5/12-1397-Techapp1.pdf). Among these 13 case-patients, 8 instances occurred in which both mother and neonate survived without sequelae; all 8 patients had received ampicillin/penicillin with or without gentamicin.

The role of gentamicin in treatment of listeriosis in pregnancy is controversial. The combination of ampicillin and gentamicin has been thought to be synergistic, although in vivo evidence of clinical benefit, compared to that of treatment with ampicillin alone, is lacking (*3*,*6*). A particular concern in pregnancy is gentamicin's poor penetration into the intracellular space, where *L. monocytogenes* is likely to reside, in the placenta (*8*). Furthermore, some concern exists that gentamicin use in pregnancy could cause fetal ototoxicity, although few such cases have been reported, and several small cohort studies have not shown this association (*9*,*10*). Our patient's child had a normal result when standard audiology testing was performed several days after delivery.

Infectious diseases clinicians will likely see patients with listeriosis in early pregnancy, given the increasing incidence of this infection in many countries and the ongoing threat of food-borne outbreaks. The collected experience from the cases reported here may be useful, particularly given the absence of high quality clinical data that support treatment recommendations for this population. Intravenous ampicillin, with or without gentamicin, effectively sterilizes the placenta and prevents maternal and fetal illness and death in cases of listeriosis in early pregnancy.

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Correction: Vol. 16, No. 12

The name of author Sri Irianti was misspelled in the article Environmental Sampling for Avian Influenza A (H5N1) in Live-Bird Markets, Indonesia. The article has been corrected online (www.wnc.cdc.gov/eid/article/16/12/10-0402).

etymologia

Acinetobacter [ascĭ-net′o-bak′tər]

From the Greek *akineto* (immobile), a genus of gram-negative paired coccobacilli that are widely distributed in nature and can cause severe primary infections in compromised hosts. *Acinetobacter* was most likely first described as *Diplococcus mucosus* in 1908. In 1954, Brisou and Prévot proposed the genus *Acinetobacter* to indicate that the bacteria were nonmotile because they lacked flagella. *Acinetobacter* are still generally described as nonmotile, but most isolates exhibit "twitching" motility.

Acinetobacter baumannii—named in honor of American bacteriologists Paul and Linda Baumann–is a nosocomial pathogen with acquired multidrug resistance that is emerging as a major concern worldwide. Motility is linked to increased virulence in bacteria such as *Pseudomonas aeruginosa* and *Dichelobacter nodosus*; however, whether motility plays a role in the virulence of *A. baumannii* remains unclear.

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In Memoriam: Alexander I. Klimov (1943–2013)

Brian W.J. Mahy

It is with great sadness that we record the untimely death of Alexander (Sasha) Klimov, PhD, ScD, who passed away on February 5, 2013, at the age of 69. Dr. Klimov began his career at the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA, following a career of great distinction at the Research Institute for Viral Preparations in Moscow, Russia, where in 1986 he became director of the World Health Organization (WHO) Collaborating Center for Molecular Biology and Genetics of Epidemic and Vaccine Influenza Virus Strains and head of the Laboratory of Genetics of RNA Viruses. Dr. Klimov came to CDC in 1991 as a visiting scientist in the Influenza Branch, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases. In 1997, he became chief of the branch's Surveillance Section, and in 2006, after a reorganization of CDC, he became chief of the Surveillance and Diagnosis Branch within the newly formed Influenza Division, National Center for Immunization and Respiratory Diseases.

During 1986–2012, Dr. Klimov participated as an invited speaker at more than 30 consultations on influenza held at WHO in Geneva. In this regard, he played a vital role in the global tracking of influenza virus strains and in the selection of appropriate vaccines for use in different regions of the world. Dr. Klimov received numerous honors, including the CDC and ATSDR Civil Service Honor Award in 1998; CDC's Charles C. Shepard Award for scientific excellence in 2001; and the James H. Nakano Award for an outstanding scientific paper, National Center for Infectious Diseases, CDC, in 1996, 2001, and 2005. Dr. Klimov was also a regular reviewer of papers submitted to Emerging Infectious Diseases, the Journal of Clinical Virology, the Journal of Virological Methods, Vaccine, Virology, and Virus Research, and he was a member of the editorial board of Voprosy Virusologii (Russia). In addition, he authored or coauthored more than 200 peer-reviewed research publications.

Alexander I. Klimov. Photograph provided by Penny Mahy.

I first met Sasha Klimov in 1981 when he visited my influenza laboratory in the Department of Pathology, University of Cambridge, Cambridge, UK, on a WHO Fellowship to study the molecular biology and genetics of influenza virus. I was immediately struck by the warmth and generosity of his personality, two characteristics that played an important role in his work as a mentor for his staff and the three PhD students whom he supervised. Sasha and I became close friends when we later met again in Moscow and at CDC. Dr. Klimov will be sorely missed by all who knew him. He leaves behind his wife, Marina Khristova, a virologist who worked on influenza virus in Moscow and who currently works in CDC's Biotechnology Core Facility Branch, as well as two children, a daughter, Tatiana, and a son, Peter, who is a composer living in Moscow.

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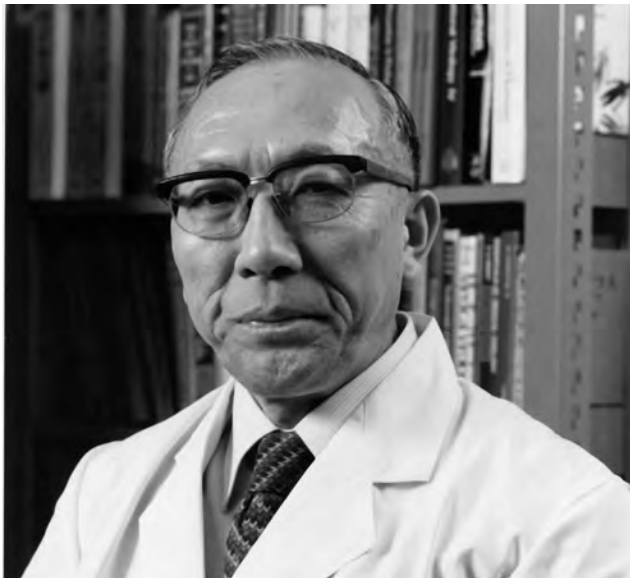
In Memoriam: Susumu Hotta (1918–2011)

Eiji Konishi and Goro Kuno

Susumu Hotta, professor emeritus of Kobe University, Kobe, Japan, passed away on November 17, 2011, at the age of 93. He was one of the early virologists trained broadly in several branches of microbiology, but he was known specifically for his dedicated research on dengue, which spanned nearly 7 decades. During his long career, he left many indelible contributions, including the first isolation of dengue virus and partially successful attempts to attenuate dengue virus for development of a vaccine.

Born in 1918 in Osaka, Japan, he spent much of his youth there. He enrolled in the Faculty of Medicine of Kyoto Imperial University (now Kyoto University), graduating with an MD degree in 1942, after which he joined the staff of the department of microbiology of the same faculty. Although his initial research interest shifted between pediatrics and neurology, he ultimately selected infectious diseases, with Ren Kimura as his advisor. This decision proved to be timely and fortuitous for his career, because in the summer of 1942, dengue outbreaks suddenly emerged in several port cities in Japan. During the next 3 summers, he investigated dengue in Nagasaki, the most severely affected city. In 1943, he succeeded in isolating dengue virus (later identified as serotype 1) from a patient for the first time.

We interrupt the chronology of Dr. Hotta's career to describe the fascinating background of this first isolation of dengue virus because Dr. Hotta rarely revealed it in public, despite its historical significance. The dengue outbreaks in Japan during the summers of 1942–1944, in the midst of World War II, were unprecedented in terms of emergence of a large-scale tropical disease outbreak in a temperate region, involvement of *Aedes albopictus* mosquitoes as the sole vectors, and the large number of patients (estimated to be as many as 2 million). In 1942, dengue outbreaks began in several port cities of Japan, where many civilians, seamen, and soldiers returned from the war-ravaged regions of the Pacific and Southeast Asia. Because these outbreaks provided a unique opportunity to study tropical disease outbreaks in a temperate region, several competing research groups, representing academic and military institutions, converged on Nagasaki, the most severely affected city, to investigate dengue.



Susumu Hotta. Photograph printed with permission from Hak Hotta.

In the summer of 1943, Dr. Hotta succeeded in isolating a strain of dengue virus (serotype 1) from the blood of a patient named Mochizuki. Dr. Hotta attributed his success to intracerebral inoculation of suckling mice, while his competitors were inoculating chick embryos or other animals. Dr. Hotta continued to visit Nagasaki every summer to pursue his research until 1945, when train service to Nagasaki was suspended because the rail track to the city had been severely damaged by Allied Forces

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bombs. Accordingly, Dr. Hotta reluctantly stayed home in Kyoto. This decision saved his life because at the School of Medicine of Nagasaki University, 3 professors who had been investigating dengue and hemorrhagic cases of dengue died instantly when the atomic bomb hit Nagasaki.

At that time in a country at war, preserving isolated virus strains was difficult, because he did not have a freeze dryer, and freezers were useless because of frequent power failures. This difficulty necessitated that he continue passages in mice. Because of shortages of almost everything in his economically devastated country, he had to feed laboratory mice with a portion of his own food ration. At one time when the supply of mice was low, his mother volunteered to keep the Mochizuki strain infectious. After injecting her with the strain, Dr. Hotta published a clinical report of the dengue syndrome in his mother. During the final days of World War II, fearing destruction of his research building and hence loss of his collection of Mochizuki and other isolated dengue virus strains, he put virus vials in a thermos bottle filled with wet ice and carried it at all times, totally unaware that Kyoto had been excluded as an Allied Forces bombing target because of its historical heritage as ancient capital of Japan.

Returning now to Dr. Hotta's career chronology, during 1953–1955, he studied dengue virus replication in cell culture under the guidance of Charles A. Evans of the University of Washington, (Seattle, WA, USA), where he received a PhD degree in 1958. In 1957, he was appointed professor of microbiology at Kobe College of Medicine, which was reorganized in 1964 to become the School of Medicine within Kobe University. He was instrumental in establishing the International Center for Medical Research at Kobe University and contributed to the promotion of international cooperative medical research with other countries, education, and training. Through cooperative international programs, he conducted a series of investigations of dengue hemorrhagic fever in Indonesia.

At Kobe University, he served as chairman of the microbiology department for many years, teaching and training many graduate students, visiting scientists, and postdoctoral fellows in a variety of subjects such as bacteriology, virology, tissue culture, and immunology. Although dengue was his major interest, he also supervised research on other arboviruses (chikungunya, Japanese encephalitis, vesicular stomatitis, and yellow fever) and on measles, rabies, hepatitis B, smallpox, shope fibroma, myxoma, and polio viruses. After retirement from Kobe University in 1982, he was appointed director of the Institute of Tropical Diseases, Kanazawa University of Medicine, Kanazawa, Japan, and continued his research on dengue until 1989. Even after his second retirement, he continued to write articles, publish books, and even coordinated full-genome sequencing of the dengue strain Mochizuki, which he had isolated in 1943.

Among Dr. Hotta's books, 2 stand out. Dengue and Related Hemorrhagic Diseases, published in 1969 by Warren H. Green, St. Louis, MO, USA, was the first book of its kind to focus on hemorrhagic dengue; and Dengue and Related Arboviruses, published in 1995 by Yukosha Printing House, Kobe, Japan, was noted for its massive compilation of nearly all Dr. Hotta's early original publications on dengue. This book, for example, reveals his discovery as early as 1949 of the anamnestic protective antibody response in secondary dengue infection. Another of his books, on tissue culture, published in 1976 in collaboration with Ren Kimura and Akio Oyama, was widely used among researchers.

Among many honors and awards that Dr. Hotta received during his life are the prestigious Saburo Kojima Award for contributions to medical science and the Order of the Rising Sun from the Government of Japan. He is survived by 3 sons, including Hak Hotta, a professor of microbiology at the Faculty of Medicine, Kobe University.

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April 2013

Emerging Viruses

Egon Schiele (1890–1918) Self-Portrait with Physalis (1912) Oil and opaque color on wood (32.2 cm × 39.8 cm) Leopold Museum, Vienna, Austria, www.leopoldmuseum.org

Titian (Tiziano Vecellio) (c. 1487/90–1576) *Sisyphus* (1548–1549) (detail) Oil on canvas (237 cm x 216 cm) Copyright Museo Nacional del Prado, Madrid, Spain. http://www.museodelprado.es/en/

Imagining Sisyphus Happy

Polyxeni Potter

"And I saw Sisyphus in agonizing torment trying to roll a huge stone to the top of a hill," wrote Homer in The Odyssey. "He would brace himself, and push it towards the summit with both hands, but just as he was about to heave it over the crest its weight overcame him, and then down again to the plain came bounding that pitiless boulder. He would wrestle again, and lever it back, while the sweat poured from his limbs, and the dust swirled round his head." This ancient myth about "the craftiest of men," whose irreverence so outraged the gods that they sentenced him to endless futile and hopeless labor, has long captured the imagination of poets but also philosophers and artists alike, among them Titian, who painted for posterity his own interpretation.

"Creation" or art is another way to express human experiences, wrote Albert Camus, in his Myth of Sisyphus (1942), a philosophical essay examining the meaning of life. The gods saw no worst punishment for Sisyphus' mischief than sentencing him to toil for no reason toward nothing, a metaphor for the human condition. Life is absurd and without meaning. In this "unintelligible and limited universe," humans have an "irrational and … wild longing for clarity." "If the world were clear, art would not exist." And, although art is not intended to explain or solve any of life's problems, it is how humans, who still possess freedom, intelligence, desire to revolt, and passion for the beauty of nature, can remain conscious of their desire to live and strive for meaning and purpose. In the philosopher's estimation, works of art are evidence of human dignity.

Titian's finest works are mythologic narratives, which he likened to visual poetry. These paintings explored human frailties with empathy and lyricism. At the behest of Mary of Hungary (1505–1558), he created a group of four paintings depicting the Damned in mythology: Tityos, Sisyphus, Tantalus, and Ixion—all condemned to perpetual torture for incurring the wrath of the gods. Only two, Tityos (his liver endlessly devoured by a vulture) and Sisyphus, have survived.

"Titian was born in Cadore, a small town on the River Piave, five miles from the pass through the Alps, to the Vecelli family, one of the noblest in those parts," wrote Giorgio Vasari in his Lives of the Artists. "And when, at the age of 10, he showed fine wit and a lively mind, he was sent to Venice to the home of an uncle, a respected citizen, who saw that the boy had a real propensity for the art of painting and who placed him with Giovanni Bellini, a skillful and very famous painter of those times." Soon he showed that "he had been gifted by Nature with all those qualities of intelligence and judgment which are necessary for the art of painting."

Venice was then the richest city in the world, where everything was traded, and no less color pigments, which were coveted by artists far and wide. The demand was so high that painting supplies were sold by specialists, the *vendecolori*. Part of an industry that encompassed members of the Venetian painters' guild, these color vendors sold only

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to painters and artisans in related trades (dyers, glass makers, decorators, potters, book publishers). Commerce with Flanders exposed Venetian artists to Flemish art and the use of oil-based paints, soon adopted by local masters, among them Giovanni Bellini and the great Giorgione, Titian's early influences. When his student's work could no longer be distinguished from his own, Giorgione was so offended that "from that time on, he never wanted to be in Titian's company or to be his friend."

The undisputed master by 1550 of the Venetian School, which included such rivals as Tintoretto, Veronese, Bassano, and Lotto, Titian would soon surpass the artistic and cultural limits of the Renaissance, and become known as the founder of modern painting, which he dominated throughout his long life, noted particularly for his inventiveness and expert use of color. "His last works," which Vasari described as "beautiful and stupendous," are "executed with such large and bold brushstrokes and in such broad outlines that they cannot be seen from close up but appear perfect from a distance." Furthermore, and to Vasari's amazement, "the pictures not only seem alive but to have been executed with great skill concealing the labor." Others said that in his visual poems, Titian embodied the greatness of Michelangelo, the grace of Raphael, and the true colors of nature.

This "sun amidst small stars," not only in Italy but around the world, freed painting from drawing and established the brushstroke as expression in its own right. Titian died during the plague epidemic in Venice. One of his sons, his right-hand man Orazio, died of the plague the same year.

Titian left no letters or other keys to his inner life or artistic vision. His more than 500 paintings support his legendary dedication to the craft. Once, while working on the portrait of Holy Roman Emperor Charles V, he dropped a brush, which the emperor promptly picked up. "Sire, one of your servants does not deserve this honor," he protested. "Titian deserves to be served by Caesar," Charles replied. Yet, this successful artist worried about money and had problems with his son, Pomponio. He spoke of "Pain and distress ... sacrifices and sweat," as he tried to set this wayward son on the path to riches. Artistic growth continued up to the time of his death, his brush becoming freer, less descriptive and more abstract. "I am finally beginning to learn how to paint."

In *Sisyphus* Titian achieved rich color effects and chiaroscuro with a limited palette, staging the human presence against a tantalizing shadow-filled space, his own earthy presentation of the myth. Like his ancestral model often depicted on ancient vases, Titian's Sisyphus is strong and vibrant, muscle-bound, determined, and sure-footed. His whole body is engaged, face strained against the load. The boulder is not pursued or pushed up the hill but carried on his shoulders, as in Ovid's Metamorphoses, a work familiar to the artist and probably his visual model.

Sisyphus [Gr. *se-sophos* = very wise or over wise] may have been the archetypal human competitor of the gods. The story has it that when Death came to take him, Sisyphus tricked and tied him up. As a result, for a time, no one could die, until Zeus intervened. Rebelliousness and love of life are essential human traits as are Sisyphean pursuits. No less so in public health and disease emergence, an endless cycle. Just as a region gets a solid grip on tuberculosis, populations shift, and the disease reappears. No sooner is a new coronavirus identified and described as SARS than a betacoronavirus with lethal respiratory and renal complications pops up in patients from countries in the Middle East. Lyssavirus, a known culprit, reinvents itself in Spain. Acinetobacter travels from North Africa to France. For all the reasons infectious diseases emerge, they can reemerge, morph into something new, and metastasize. And public health workers keep pushing the rock up the hill.

Despite darkness and futility in the world, Titian imagined Sisyphus thundering upward at his task. Despite the absurd, Camus surmised that "the struggle itself toward the heights is enough to fill one's heart," concluding that one "must imagine Sisyphus happy." And in public health, where monumental effort sometimes brings incremental improvement, success is still measured by tying up Death.

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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 70% passing score) and earn continuing medical education (CME) credit, please go to 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 New Users: Free Registration link on the left 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/category/2922.html. 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:

Transmission of *Mycobacterium tuberculosis* Beijing Strains, Alberta, Canada, 1991–2007

CME Questions

1. You are seeing family of 5 who emigrated from China one year ago. The father of the family was recently diagnosed with pulmonary tuberculosis (TB), and you are worried about the potential spread of the infection, particularly with virulent strains of *Mycobacterium tuberculosis*. What should you consider in general regarding the epidemiology of the Beijing lineage of *M. tuberculosis*?

A. It accounts for 13% of all TB strains globally
B. It accounts for less than 2% of cases of TB in immigrant-receiving countries with low rates of infection
C. It is rarely resistant to treatment
D. The Beijing strain is less virulent compared with other TB strains in animal models

2. Which of the following characteristics was most associated with TB infection with the Beijing strains in the current study?

A. Higher proportion of foreign-born patients
B. Younger age
C. More likely to have a positive sputum smear
D. Higher rates of drug resistance

3. What should you consider regarding the transmission of Beijing vs non-Beijing strains of TB in the current study?

A. The Beijing strains were associated with higher rates of clustered cases
B. The Beijing strains were associated with similar rates of clustered cases compared with non-Beijing strains
C. The Beijing cases were only more likely to be clustered among foreign-born patients
D. The Beijing cases were only more likely to be clustered among drug-resistant strains

4. Which of the following factors was most positively associated with a higher rate of secondary cases of TB in the current study?

A. Beijing lineage
B. Age over 64 years in the index case
C. Positive sputum smear in the index case
D. Foreign-born patient in the index case

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue June 2013

Public Health Lessons from Severe Acute Respiratory Syndrome a Decade Later

Prospects for Emerging Infections in East and Southeast Asia 10 Years after Severe Acute Respiratory Syndrome

New Delhi Metallo-β-Lactamase–producing *Enterobacteriaceae*, United States

Blood-borne Viral Infections in Refugee Children from War and Transition Zones

Pandemic Influenza Planning, United States, 1976–2008

Cell Culture and Electron Microscopy for Identifying Viruses in Diseases of Unknown Etiology

Lessons, Progress, and Gaps 10 Years after SARS

A Systematic Review on the Worldwide Occurrence of Tuberculosis Caused by *Mycobacterium bovis* Infections in Humans

Contribution of Travelers to Influenza Epidemiology

Novel *Mycobacterium tuberculosis* Complex Isolate from a Wild Chimpanzee

Effect of Winter School Breaks on Incidence of Influenza-like Illness, Argentina, 2005–2008

Foodborne Botulism in Canada, 1985–2005

Characteristics of Group A *Streptococcus* Strains Circulating during Scarlet Fever Epidemic, Beijing, China, 2011

Spatiotemporal Dynamics of Dengue Epidemics, Southern Vietnam

Invasive Disease Caused by *Haemophilus Influenzae* Serotype A, Alaska, USA

Active Surveillance for Influenza A Virus in Swine, Midwestern United States

Transmission Potential of Rift Valley Fever Virus over Course of Epidemic, South Africa, 2010

Upcoming Infectious Disease Activities

2013

May 1–4, 2013

The Society for Healthcare Epidemiology of America (SHEA)

Spring 2013 Conference

Atlanta, GA, USA

http://shea2013.org

September 5–10, 2013

Options for the Control of Influenza VIII

Cape Town, South Africa

http://www.isirv.org

September 10–13, 2013

ICAAC 2013
(Interscience Conference on Antimicrobial Agents and Chemotherapy)

Denver, Colorado, USA

http://www.icaac.org

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.

EMERGING INFECTIOUS DISEASES

Complete list of articles in the June issue at:

http://www.cdc.gov/eid/upcoming.htm

EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

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

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

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

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

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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.

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.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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