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July 2009

Vector-borne Infections

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EMERGING INFECTIOUS DISEASES

July 2009

On the Cover

Pieter Bruegel the Elder
(c. 1525–1569)
The Fall of the Rebel Angels (1562)
Oil on panel (117 cm × 162 cm)
Musées Royaux des Beaux-Arts
de Belgique, Brussels.
Photo: Lefevre

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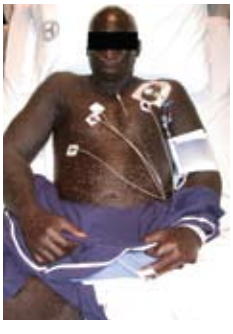

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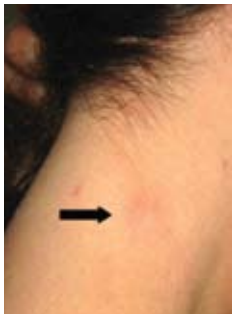

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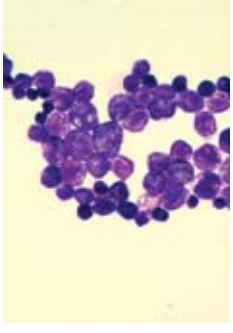

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Cluster of Sylvatic Epidemic Typhus Cases Associated with Flying Squirrels, 2004–2006

Alice S. Chapman,[1] David L. Swerdlow, Virginia M. Dato, Alicia D. Anderson, Claire E. Moodie, Chandra Marriott, Brian Amman, Morgan Hennessey, Perry Fox, Douglas B. Green, Eric Pegg, William L. Nicholson, Marina E. Eremeeva, and Gregory A. Dasch

CME ACTIVITY

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Learning Objectives

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

- Describe the transmission of sylvatic epidemic typhus
- Specify the most likely exposure to *Rickettsia prowazekii* among cases in the current series
- List common symptoms and signs of sylvatic epidemic typhus
- Describe the management of suspected sylvatic epidemic typhus

Editor

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In February 2006, a diagnosis of sylvatic epidemic typhus in a counselor at a wilderness camp in Pennsylvania prompted a retrospective investigation. From January 2004 through January 2006, 3 more cases were identified. All had been counselors at the camp and had experienced febrile illness with myalgia, chills, and sweats; 2 had been hospitalized. All patients had slept in the same cabin and reported having seen and heard flying squirrels inside the wall adjacent to their bed. Serum from each patient had evidence of infection with *Rickettsia prowazekii*. Analysis of blood and tissue from 14 southern flying squirrels trapped in the woodlands around the cabin indicated that 71% were infected with *R. prowazekii*. Education and control measures to exclude flying squirrels from housing are essential to reduce the likelihood of sylvatic epidemic typhus.

Sylvatic epidemic typhus, hereafter referred to as sylvatic typhus, is a rare but potentially lethal zoonotic exanthematous disease caused by *Rickettsia prowazekii*. It is associated with a cycle of infection involving flying squirrels and their ectoparasites and secondary transmission to humans. Illness in humans is characterized by fever, myalgia, severe headache, and rash. Historically, classic louseborne epidemic typhus, caused by the same organism, has caused large epidemics where conditions were favorable for person-to-person spread of body lice.

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Surveillance for epidemic typhus was discontinued in the United States in the 1950s because the illness had not been reported in decades and prevalence of body lice infestation in this country has been low (*1*). However, in 1975, the southern flying squirrel, *Glaucomys volans*, was found to be naturally infected with *R. prowazekii* (*2*). Later studies demonstrated serologic evidence of human *R. prowazekii* infection associated with flying squirrels in the eastern United States (*1*,*3*,*4*). Fleas and lice carried by the squirrels become naturally infected (*1*,*5*,*6*) and may be responsible for transmission (*7*); however, the exact mechanism of transmission remains unknown. From 1976 through 2002, a total of 41 cases of human *R. prowazekii* infection were documented in persons who had no reported contact with body lice or persons infested with lice (*3*,*4*,*7*–*10*). Almost all cases occurred in the eastern United States. Approximately one third of the patients had confirmed contact with flying squirrels or their nests before disease onset (*4*,*7*); the remaining patients had no identified association with flying squirrels.

On February 23, 2006, serologic testing performed at the Centers for Disease Control and Prevention (CDC) identified a case of sylvatic typhus in a 31-year-old counselor at a therapeutic wilderness camp for troubled youth in Pennsylvania (case-patient 1). A review of the Pennsylvania electronic disease surveillance system found that in December 2004 another counselor from the same camp (case-patient 2) had become ill with fever, chills, and sweats and that serologic testing at a local laboratory had been suggestive of sylvatic typhus. During a 25-year period (1976−2001), only 2 other cases of sylvatic typhus associated with flying squirrels had been reported in Pennsylvania (*7*). Laboratory and epidemiologic investigations of humans and animals associated with the camp were conducted to identify additional sylvatic typhus cases at the camp, risk factors for infection, and prevalence of *R. prowazekii* in the natural host. A total of 4 cases of sylvatic typhus were found to have occurred during the 24-month period of January 2004–January 2006. All were linked epidemiologically to a specific cabin and bed at the Pennsylvania camp. The investigation also found laboratory evidence of *R. prowazekii* infection in the population of flying squirrels at the camp.

Methods

Epidemiologic Investigation

We defined a suspected case of sylvatic typhus as fever (≥38°C) plus ≥1 of the following: headache, myalgia, confusion, rash, or photophobia, with illness onset prior to March 1, 2006, in a staff member or student at the camp. A confirmed case of sylvatic typhus was defined as a case that met the definition of a suspected case plus had immunoglobulin (Ig) M and/or IgG reactive with whole-cell *R. prowazekii* antigen at a dilution >1:64, according to immunofluorescence antibody (IFA) assay performed at CDC (*11*). Persons who met the definition for having a suspected case but who had negative serologic results for *R. prowazekii* were not considered to have a confirmed case of sylvatic typhus. Interviews were conducted to ascertain exposure history, and medical records were reviewed. In March 2006, to identify other possible cases of sylvatic typhus and to assess risk factors for infection, a standardized questionnaire was administered to the camp staff and serum samples were obtained. The questionnaire gathered information about job classification, length of employment, cabin sleeping history, and contact with flying squirrels or their nests. Medical records of enrolled students (typically at the camp for 9–18 months) were assessed to identify illness suggestive of sylvatic typhus occurring during their residence. Records of students discharged from the program before February 2006 were not available for review. Data analyses were performed by using Epi Info 2000 (*12*). Odds ratios, confidence intervals, and p values were calculated by using SAS version 9.1.3. (SAS Institute, Inc., Cary, NC, USA) (*13*).

Environmental Investigation

During the winter (October–March), staff and students had lived in 1 of 6 cabins (cabins A–F); during the rest of the year, they had slept in tents (Figure). All 6 cabins were inspected for evidence of animal infestation, including flying squirrels, and for openings where flying squirrels could enter the cabins. In March 2006, baited live trapping using Sherman box traps (H.B. Sherman Traps, Tallahassee, FL, USA) was conducted on 5 consecutive nights throughout the camp. Trapping sites included a tent site occupied only during summer; sites of cabins A, B, and F; and 2 nearby sites not directly associated with the cabins.

Blood was obtained from trapped flying squirrels for detection of IgG (heavy plus light chain) polyvalent antibodies against *R. prowazekii* by IFA. Serologic testing was performed at CDC with an anti−flying squirrel fluorescein-isothiocyanate conjugate prepared in-house. Samples were first screened at 1:32 dilution; any reactive samples were titered to end point. Necropsies were performed and tissues were collected, frozen on dry ice, and kept at –70°C before further testing. DNA was prepared from whole blood and heart tissues by using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. PCRs to amplify a fragment of 17-kDa protein antigen were carried out in a total volume of 25 μL by using Taq Master Mix (QIAGEN) in a Gradient Mastercycler (Eppendorf, Westbury, NY, USA). Details of the PCR setup and primer sequences are described elsewhere (*14*). Identity of the PCR amplicon was confirmed by direct sequencing performed using an ABI PRISM 3.0 BigDye Terminator Cycle

Sequencing kit as recommended by manufacturer (Applied BioSystems, Foster City, CA, USA). Nucleotide sequences generated during this study were submitted to the NCBI GenBank.

Results

Of 66 camp staff members on site during the March 2006 investigation, 45 (68%) completed a questionnaire; 33 of the 45 (73%) provided a serum sample, including 17 staff members who met the case definition for suspected sylvatic typhus. Five additional staff members reported having had febrile illness since employment at the camp but declined testing; 2 of them met the definition for suspected sylvatic typhus. Serologic testing confirmed 4 (12%) cases of sylvatic typhus among the 33 tested staff members, including case-patients 1 and 2, who were identified at the start of the investigation (Table 1). The remaining 13 staff members who met the definition of having a suspected case but who had no serologic evidence of *R. prowazekii* infection were classified as non–case-patients. Two persons who had suspected cases but did not undergo serologic testing could not be further classified and were grouped with other survey-only respondents for the purpose of analysis.

Case Reports

Case 1

On January 7, 2006, a 31-year-old man from western Pennsylvania became ill with high fever (39.6°C), chills, sweats, headache, and joint and muscle aches. The next day, he was admitted to the hospital. During his hospitalization, he continued to have fever and muscle aches, and laboratory test results were positive for mononucleosis and influenza A. He was discharged on January 17 but continued to have headache and fever. After hearing of a case of sylvatic typhus at the camp where he worked the previous year, he requested testing from his hospital clinician. On January 24, he was reevaluated and serum was obtained for sylvatic typhus testing. He was given oral doxycycline, 100 mg twice a day for 10 days; his signs and symptoms resolved. The man had lived in cabin A and stated that although he had never handled a flying squirrel, he had heard them frequently in the wall next to his bunk (bunk B) (Figure).

Case 2

On December 27, 2004, a 39-year-old man from southwestern Pennsylvania had fever (40.3°C), chills, sweats, headache, and muscle and joint pain. He later became confused and was admitted to the hospital on January 4, 2005. At the time of admission, he was febrile and dehydrated. He was treated with azithromycin and ciprofloxacin but did not improve. When he told clinicians that he worked at a wilderness camp and had been bitten by fleas, he was administered a regimen of intravenous doxycycline for 3 days. His fever subsequently resolved; he was discharged from the hospital in early January and given oral doxycycline, 100 mg twice a day for 7 days. He visited his primary care provider in February 2005. IgG and IgM tests performed at a commercial laboratory on serum collected on February 1, 2005, showed qualitatively positive results for typhus group–specific antibodies. The man had lived in cabin A at the time of his illness. He reported having seen a flying squirrel 1 time in the main living area of the cabin and seeing squirrels in the walls next to his bunk. He also reported feeding the squirrels through wide gaps in the wall boards next to his bunk (bunk B) and being bitten by insects he recognized as fleas in his bunk at least 1 time the previous year.

Case 3

In January 2004, a 26-year-old man from Pennsylvania had high fever (39.4°C), chills, sweats, muscle aches, nausea, and vomiting. The man did not seek medical attention and recovered within a week. He worked as a counselor at the camp in Pennsylvania and, at the time of his illness, was living in cabin A. He reported that he had seen animals inside gaps in the wall next to his bunk (bunk B) and had seen a flying squirrel in the main living area of the cabin on 1 occasion.

Table 1. Characteristics of patients with flying squirrel–associated sylvatic typhus, Pennsylvania, USA, 2004–2006*

Patient no. (age, y)†	Date of illness onset	Exposure‡		Serologic titer (date)		Clinical sign§			Hospitalized
		Touched flying squirrel	Slept in bunk B	IgG	IgM	Fever, °C	Headache	Photophobia	
1 (31)	2006 Jan	No	A	512 (2006 Feb)	4,096 (2006 Feb)	39.6	Yes	No	Yes
2 (39)	2004 Dec	Yes	F	1,024 (2006 Mar)	512 (2006 Mar)	40.3	Yes	Yes	Yes
3 (26)	2005 Jan	No	F	128 (2006 Mar)	ND	39.4	No	No	No
4 (26)	2005 Dec	No	S	256 (2006 Mar)	ND	39.8	Yes	Yes	No

*Ig, immunoglobulin titer against *Rickettsia prowazekii;* ND, not done.
†All patients were male.
‡All patients lived in cabin A. A, always; F, frequently; S, sometimes.
§All patients had chills and muscle pain but not rash.

Case 4

In late December 2005, a 26-year-old man from Pennsylvania had high fever (39.8°C), severe headache, muscle and joint pain, chills, sweats, and photophobia. He did not seek medical attention and recovered after 2 weeks. He worked as a counselor at the camp and, at the time of his illness, lived in cabin A. He reported having seen and heard animals inside the wall next to his bunk (bunk B) many times and attempting to seal gaps in the wall with foam caulking spray. Although he had never touched a flying squirrel, he had seen one traversing a roof truss inside cabin A, had handled nesting material, and had been bitten by insects he recognized as fleas while living in the cabin before his illness.

The median age of all 45 questionnaire respondents was 34 years (range 21–69 years); 97% were white, and 84% were male. The median length of employment at the camp was 2 years (range 1 month–31 years). Selected characteristics reported are summarized in Table 2. Of 45 (69%) respondents, 31 reported seeing gaps between interior wall boards in all cabins. Gaps were most frequently observed in cabin A (24/31; 77%), followed by cabins D (13/31; 42%), F (12/31; 38%); E (10/31; 32%); and B and C (each 9/31; 29%) (Figure). Seeing flying squirrels in a cabin was reported by 15 (33%) respondents; however, 7 could not recall which cabin. Among the 8 who recalled the location, 5 (63%) reported seeing them in cabin A and 1 each recalled seeing them in cabins B, C, and D.

The 4 confirmed case-patients had lived in cabin A for a significantly longer cumulative period (median 17 months) than non–case-patients (median 2 months; p = 0.04). Bunk

Table 2. Selected characteristics of surveyed staff members at wilderness camp in Pennsylvania, March 2006*

Characteristic	Case-patients (n = 4)	Non–case-patients (n = 29)	Case-patients surveyed† (n = 12)
Median age, y‡	28.5 (range 26–40)	38.7 (range 21–69)	35 (range 23–63)
Gender			
Male‡	4 (100)	23 (79)	11 (92)
Female	0	6 (21)	1 (8)
Job classification			
Counselor‡	4 (100)	14 (48)	7 (58)
Maintenance	0	3 (10)	1 (8.3)
Administrative	0	5 (17)	1 (8.3)
Support staff	0	5 (17)	1 (8.3)
Night security	0	1 (3.4)	2 (17)
No answer	0	1 (3.4)	0
Employment, y‡	2.5 (range 1.8–3.9)	2.7 (range 0.1–30.8)	1.1 (range 0.2–26.3)
Ever slept in cabins			
Yes‡	4 (100)	19 (66)	8 (66.6)
No	0	10 (34)	3 (25)
Don't know	0	0	1 (8.3)
Contact with (touching) flying squirrel			
Yes‡	1 (25)	7 (24)	1 (8)
No	3 (75)	20 (69)	11 (92)
Don't know	0	1 (3.4)	0
No answer	0	1 (3.4)	0
Contact with (touching) flying squirrel nests§			
Yes	2 (50)	3 (10)	0
No	1 (25)	17 (58.6)	10 (83)
Don't know	1 (25)	8 (28)	2 (17)
No answer	0	1 (3.4)	0
Ever slept in cabin A			
Yes‡	4 (100)	13 (45)	4 (33)
No	0	16 (55)	8 (67)
Ever slept in bunk B in cabin A			
Yes¶	4 (100)	9 (69)	2 (50)
No	0	3 (23)	1 (25)
No answer	0	1 (7.6)	1 (25)
Cumulative months in cabin A#	17.00 (range 8–43)	2.00 (range 0–13)	0.25 (range 0–30)

*Data given as frequency (%) except as indicated.
†Responses of staff members who declined serologic testing. Remaining respondents classified as case-patients or non–case-patients, according to results of serologic testing for *Rickettsia prowazekii* and patients' medical histories.
‡These characteristics did not demonstrate statistical difference between case-patients and non–case-patients.
§Odds ratio 11.3 (95% confidence interval 0.51–474) for association between contact with flying squirrel nests and sylvatic typhus.
¶Association with sleeping in bunk B of cabin A significant at p = 0.02.
#Significant at p = 0.04.

B had been slept in by all 4 case-patients but by only 9 of 28 non–case-patients (1 non–case-patient unknown; p = 0.02, by Fischer exact test). From January 2004 through January 2006, 8 (18%) staff members lived primarily in cabin A, and other staff members occasionally slept in cabin A.

Although the students living in cabin A were not tested for typhus, a review of their medical records indicated that none had sought medical care for a febrile illness at any time during their enrollment. Of 43 students whose records were reviewed, 7 (16%) had experienced febrile illness compatible with sylvatic typhus since their arrival at the camp. These students resided in cabins D, E, and F. All 6 (86%) who were tested for evidence of *R. prowazekii* infection had negative results.

Environmental Investigation

At the time of the March 2006 investigation, counselors and students were living in 5 of 6 cabins located on a 1,500 acre tract of land in southwestern Pennsylvania (Figure). The cabins were widely separated across the tract. Each site was occupied by ≈2 counselors and 10 students, who worked and attended school at the site and interacted with each other during events and meals. The land is mountainous and covered with a forest of mixed northern hardwood, predominantly oak, maple, black cherry, and yellow poplar. The cabins consisted of rough-cut wood planking on wooden stud construction. The roof was covered with asphalt shingles. Inside, 8–10 wood-framed bunk beds (for students) were primarily located against the cabin walls, surrounding a centrally placed wood-burning stove. A small alcove in each cabin, containing 2 bunk beds each set against a wall, was designated for counselors' use. Bathing facilities were located in a separate building. Exterior walls were covered in horizontal, planed lumber. The inside walls were constructed of vertical planed lumber. Fiberglass insulation batting was installed in the hollow spaces in the wall.

When examined, the cabins at sites A, C, D, and F had evidence of deer mice and flying squirrel infestation, including nesting material protruding from the walls and ceiling and damaged or chewed insulation in walls adjacent to counselor bunk A (cabin C), counselor bunk B (cabin A), and student bunks (cabin D). A large opening providing access to the exterior of the cabin was present on the wall above the counselor bunk B in cabin A. In April 2006, camp staff removed the interior wooden planks from the wall adjacent to bunk B in cabin A and confirmed the presence of flying squirrel nesting materials and extensive damage to insulation inside the wall.

Evidence of *R. prowazekii* Infection in Trapped Flying Squirrels

Trapping efforts obtained 14 southern flying squirrels from several outdoor sites throughout the camp, including the site where cabin A was located and a field site adjacent to a state park (Figure; Table 3). Of the 14 squirrels, 8 (57%) had antibodies to *R. prowazekii*, 6 with end-point titers from 32 to 1,024 (Table 4). These 8 squirrels had been captured at a tent site in the camp woodlands (n = 3), field sites not associated with cabins (n = 4), and a path leading to cabin A (n = 1). *R. prowazekii* DNA was detected in specimens (2 hearts and 3 whole blood samples) from 5 (36%) squirrels. Of the 14 squirrels, 3 showed both serologic and molecular evidence of infection and 12 hosted fleas or lice (data not shown).

Discussion

Our investigation found that transmission of sylvatic typhus to humans occurred during 3 consecutive winters at the camp. All 4 cases among counselors were epidemiologically linked to cabin A. All had slept in a specific bunk, bunk B, next to a wall that had evidence of flying squirrel infestation. Other counselors sleeping in the same cabin but without direct exposure to bunk B had no evidence of infection. The finding that 10 (71%) of 14 flying squirrels collected at multiple sites showed evidence of *R. prowaze-*

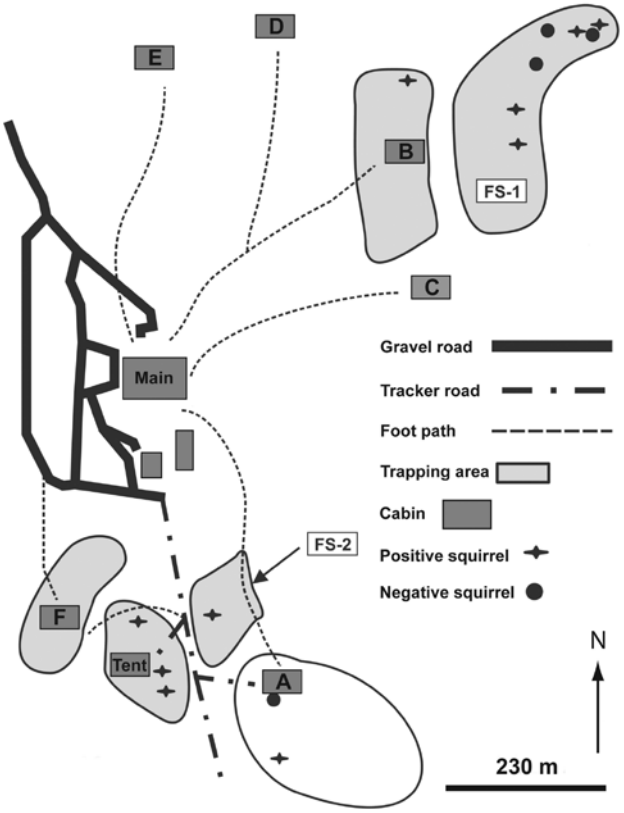

Figure. Wilderness camp, Pennsylvania, USA, showing areas where flying squirrels were trapped over a 5-day period during March 2006 for *Rickettsia prowazekii* testing. Cabins and tent sites are designated by letters A, B, C, D, E, F, and Tent. Field sites are designated FS-1 and FS-2.

Table 3. Total arboreal trapping effort and captures of flying squirrels, wilderness camp, Pennsylvania, March 2006*

Site	Traps	Trap-nights†	Captures	%‡
Cabin A	36	126	2	1.5
Cabin B	30	120	1	0.8
Cabin F	23	100	0	0
Tent	32	94	3	3.2
FS-1	30	90	7	7.8
FS-2	15	60	1	1.7
Total	166	500	14	2.8

*FS, field site.
†Trap-night = 1 trap/1 night of effort. 5 traps for 1 night = 5 trap-nights.
‡No. captures divided by no. trap-nights.

kii infection indicates that the pathogen is well established among these squirrels.

Although inhalation and transdermal or mucous membrane exposure to infected louse feces are well-established routes of transmission during epidemics of louse-borne typhus, the mechanism by which *R. prowazekii* is transmitted from flying squirrels to humans is not well understood. The lack of detectable antibodies to *R. prowazekii* in household members of documented sylvatic typhus case-patients (*6*,*8*) has been used to support the hypothesis that risk for sylvatic typhus in the absence of direct exposure to flying squirrels and their nests is low (*9*) and thus may explain why human disease has been reported only sporadically. The cluster of cases described here suggests that where repeated and prolonged close exposure to flying squirrels and their nests occurs, potential for transmission can be high. Only 1 case-patient described here reported direct contact with flying squirrels; however, all case-patients slept for many nights next to a wall that was continuously inhabited by flying squirrels. Nesting material, dander, arthropod feces, or ectoparasites may have been introduced to the living area of the cabin through the many openings in this cabin wall and provided a source of infection for those sleeping in the bunk adjacent to the wall but not elsewhere in the cabin. Because cabins at this camp were occupied continuously during the fall and winter months by the same group of counselors, epidemiologic linking of several infections occurring at different times during consecutive winters was possible. No identified cases occurred during the summer when staff and students lived outdoors in tents, further suggesting that exposure was highly focal to cabin A.

During spring of 2006, cabin A was vacated by staff and students for remediation. External openings were closed by using wire hardware cloth to exclude squirrel entry. The inside wall boards of the cabin were removed, insulation was replaced, and new wall boards were installed. The entire cabin was professionally treated to eliminate ectoparasites. Staff and students were educated about sylvatic typhus and the need to avoid contact with flying squirrels and their nests. Since then, no additional cases of sylvatic typhus have been identified.

In contrast to classic epidemic typhus, which typically causes severe disease and mortality rates up to 4% despite antimicrobial drug therapy, fatal cases of typhus associated with flying squirrels have not been reported (*15*). Although flying squirrel isolates of *R. prowazekii* are reported to have biological, biochemical, and molecular properties similar to those of other typhus isolates (*16*–*18*), most cases of sylvatic typhus in the United States are apparently less severe than classic louse-borne epidemic typhus. All 4 patients with confirmed cases reported here had fever, headache, and malaise typical of sylvatic typhus; however, only 2 (case-patients 1 and 2) were hospitalized. None of the case-patients reported having had a rash with their illness. Rash has been reported for only half of patients with flying squirrel–associated typhus (*10*) and cannot be considered a reliable sign of the disease. Milder disease associated with sylvatic typhus may result from better nutritional and overall health status of most persons in the United States compared with those in populations affected by louse-borne epidemic typhus during war or other catastrophe. Because sylvatic typhus was not initially considered for any case-patients reported here, despite their occupational history, diagnosis and treatment were delayed. Given the nonspecific signs and symptoms associated with sylvatic typhus, the disease is likely underdiagnosed.

Clinicians should consider sylvatic typhus when evaluating a patient with compatible signs and symptoms and should inquire about potential exposures to flying squirrels. Clinicians who suspect sylvatic typhus on the basis of clinical presentation and history of potential exposure should empirically treat with doxycycline and not withhold treatment pending laboratory confirmation by serologic testing. Submission of blood or skin punch biopsy samples (when rash is present) for PCR analysis may provide options for earlier diagnosis, but these assays are not widely available.

Table 4. Serologic and PCR testing results of trapped flying squirrels, wilderness camp, Pennsylvania, March 2006*

Squirrel (ID)	Site trapped	IgG titer† to *Rickettsia prowazekii*	PCR result (specimen)
1 (P19)	Tent	512	– (blood)
2 (A59)	FS-1	32	– (blood)
3 (A60)	FS-1	<16	– (heart, blood)
4 (P1)	Tent	64	+ (heart)
5 (P28)	Tent	32	– (blood)
6 (A41)	FS-1	128	– (blood)
7 (A53)	FS-1	<16	– (blood)
8 (C4)	Cabin A	<16	– (blood)
9 (A43)	Cabin B	<16	+ (heart)
10 (A26)	FS-1	1,024	+ (blood)
11 (A56)	FS-1	<16	– (blood)
12 (A60h)	FS-1	<16	+ (blood)
13 (H12)	FS-2	64	+ (blood)
14 (C52)	Cabin A	128	– (blood)

*Ig, immunoglobulin; ID, identification number.; FS, field site.
†Heavy plus light chain.

Serologic confirmation of infection is based on 4-fold IgG titer increases between acute and convalescent samples; both IgM and IgG can persist for years after infection with *R. prowazekii*.

Because southern flying squirrels are distributed throughout the eastern United States, other woodland settings frequented by humans during winter may present a similar risk for sylvatic typhus. Parks and campgrounds that maintain rental cabins should be aware of risks associated with flying squirrels and take steps to exclude these animals from structures occupied by humans by sealing openings at attic vents and around roof joists with heavy-gauge screen or similar products. In addition, use of repellents may keep arthropods from biting humans, and premise sprays may be useful for reducing arthropods in building structures. Squirrel removal without concomitant arthropod control is not recommended because the presence of potentially infected external parasites may increase the risk for disease transmission to humans.

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Increased Host Species Diversity and Decreased Prevalence of Sin Nombre Virus

Laurie J. Dizney and Luis A. Ruedas

Emerging outbreaks of zoonotic diseases are affecting humans at an alarming rate**.** Until the ecological factors associated with zoonoses are better understood, disease emergence will continue. For Lyme disease, disease suppression has been demonstrated by a dilution effect, whereby increasing species diversity decreases disease prevalence in host populations. To test the dilution effect in another disease, we examined 17 ecological variables associated with prevalence of the directly transmitted Sin Nombre virus (genus *Hantavirus*, etiologic agent of hantavirus pulmonary syndrome) in its wildlife host, the deer mouse (*Peromyscus maniculatus*). Only species diversity was statistically linked to infection prevalence: as species diversity decreased, infection prevalence increased. The increase was moderate, but prevalence increased exponentially at low levels of diversity, a phenomenon described as zoonotic release. The results suggest that species diversity affects disease emergence.

During the past 60 years, the number of emerging pathogens affecting humans has substantially increased (*1*). Of these emerging infectious diseases, 62% are zoonotic (*2*), meaning they are naturally hosted by, and persist in, wildlife but also affect human populations. The ecological factors associated with zoonotic disease emergence are likely complex and are poorly understood. Most often, because of limited time, resources, and the exigencies of the situation, outbreak investigations of emerging diseases seek only to discover the pathogen responsible for the disease in humans. But ecological studies are of critical importance to long-term containment of zoonotic disease emergence; they are the only way to ascertain the wildlife source of the disease, the dynamics of the host–pathogen relationship, and the ecological factors associated with an outbreak. Knowledge of all these factors is needed to proactively protect the public from zoonotic diseases; without this knowledge, new diseases will continue to emerge. The worldwide distribution of these largely zoonotic diseases suggests a globally distributed mechanism for their emergence.

Anthropogenic factors—including pollution, land-use conversions, and climate change—likely contribute to disease emergence by several mechanisms (*3*), one of which has been hypothesized to be decreased species diversity. The number of species currently being lost, as well as the rate of species loss, is unprecedented (*4*); these losses generally have negative effects on ecosystem functioning (*5*,*6*). It likely is not coincidental that areas where many zoonoses are emerging among humans are the same areas where loss of species is accelerating, e.g., Central Africa (Ebola, monkeypox, Marburg virus), West Africa (Lassa virus, HIV-2), Southeast Asia (Nipah virus, severe acute respiratory syndrome, avian influenza), and South America (dozens of strains of hantaviruses and arenaviruses).

Lyme disease, a vector-borne zoonosis, is affected by loss of species by a process known as the dilution effect (*7*), whereby increasing species diversity decreases disease prevalence by diluting the availability of competent hosts with increased numbers of noncompetent hosts. Little research on the dilution effect has been carried out beyond its effect on Lyme disease (*8*), yet the global implications of the phenomenon—if the effects are applicable to other types of diseases and transmission dynamics—could have substantial and enduring effects on human health and conservation.

Hantaviruses provide a model system in which to test the dilution effect in directly transmitted zoonoses. Since their initial discovery in the Western Hemisphere in 1982, several dozen hantavirus strains have been found, each hosted by a unique rodent species (*9*); novel hantaviruses have

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recently been discovered in shrews (*10*,*11*). Natural hosts are asymptomatic and chronically infected; intraspecies spread is hypothesized to be through bites (*12*). Humans become infected with hantavirus by inhaling aerosolized excreta from infected rodents (*13*). Occasionally hantavirus pulmonary syndrome (*14*) develops; this syndrome has a mortality rate of almost 40% and no prophylaxis, treatment, or cure (*15*). Most of the 506 confirmed cases in the United States have been caused by Sin Nombre virus (SNV). Studies have found that low diversity ecosystems dominated by the rodent hosts for 3 distinct hantaviruses had high infection prevalence in the host (*16*,*17*), suggesting a role for species diversity. Although the mechanism of disease dilution would differ in directly transmitted zoonoses (e.g., hantaviruses), as opposed to vector-borne diseases, a dilution effect could occur if 1) individuals of the host species remain as species diversity decreases, 2) the disease is spread within the host species through direct encounters (such as biting), and 3) presence of other species causes encounters among the host species to decrease.

Other ecological factors could affect the number of intraspecific deer mouse (*Peromyscus maniculatus*) encounters, including increased density of deer mice and vegetative factors that lead to variation in population numbers (e.g., available cover and forage) (Table 1). Some studies have found high SNV prevalence in host populations when deer mice densities were high (*18*,*19*). However, although the concept of density-dependent transmission is not unique to hantaviruses, its applicability to the deer mouse–SNV system has been elusive. SNV prevalence also has been shown to vary with habitat characteristics and quality (*15*,*18*,*19*), although interpretation of this variation has been difficult because SNV prevalence varies as much within as among habitat types (*20*).

In this study we examined small mammal populations in 5 forested sites over a 3-year period, October 2002 through September 2005. We monitored mammal species diversity, deer mouse densities, and SNV infection prevalence in the mammals to test the hypotheses that 1) areas of higher mammal species diversity would exhibit lower prevalence of SNV infection in host populations, 2) areas of higher host density would contain higher infection prevalence of SNV in the host populations, and 3) vegetative factors could be related to prevalence of SNV infection among deer mice.

Materials and Methods

Sites

We sampled small mammals at 5 sites in and around Portland, Oregon, USA: site 1, Forest Park (45.5916°N, 122.7983°W); site 2, Tryon Creek State Park (45.4337°N, 122.6690°W); site 3, Powell Butte Portland City Park (45.4837°N, 122.5059°W); site 4, Oxbow Metro Regional Park (45.4879°N, 122.2970°W); and site 5, Tualatin River National Wildlife Refuge (45.3957°N, 122.8305°W). Detailed site descriptions can be found in Dizney et al (*21*).

Trapping and Blood Sampling

To sample as many different mammal species as possible, we set up a trapping web 200 m in diameter (*22*) at each site and used 4 trap types: Sherman (H.B. Sherman Traps, Tallahassee, FL, USA), handmade wire mesh, Tomahawk (Tomahawk Live Trap Co., Tomahawk, WI, USA), and pitfall. Each station included an aluminum folding Sherman live trap and a custom-built mesh live trap (*23*) of similar dimensions (7.6 cm × 8.9 cm × 22.9 cm). Two sizes of Tomahawk live traps were used to trap larger animals; a 61 cm × 17.8 cm × 17.8 cm trap was placed at each 50-m trap station, and a 91.4 cm × 25.4 cm × 30.5 cm trap was placed at each 100-m trap station. Pitfall traps were made by using a 19-L bucket (30-cm

Table 1. Vegetative factors measured within each site and their transformations, Portland, Oregon, USA, October 2002–September 2005

Habitat	Description	Transformation
Tree cover	% Plot covered with trees	$\log_{10}$ +1
Shrub cover	% Plot covered with shrubs	Square root
Bryophyte	% Plot covered with bryophytes	Square root
Bare ground	% Plot that is bare ground	$\log_{10}$ +1
Bare ground and litter	% Plot that is bare ground and bare ground covered with litter	Square root
Ground cover	% Ground of plot that has any cover, including plants, logs, litter	None
Plant ground cover	% Plot that has only plant ground cover	None
Coarse woody debris	% Plot that is logs, stumps, snags	$\log_{10}$ +1
Trees	No. all trees	$\log_{10}$ +1
Large trees	No. trees >25 cm circumference	$\log_{10}$ +1
Maximum tree height	Tallest tree in plot	None
Total shrubs	No. all shrubs	$\log_{10}$ +1
Small shrubs	No. shrubs <99 cm tall	Omitted
Large shrubs	No. shrubs >100 cm tall	Omitted
Plant species	No. plant species	None

diameter, 36-cm depth) with a lid for rain and predator cover suspended ≈8 cm above ground to enable access by small animals (*24*); pitfall traps were placed at each 20-, 50-, and 100-m trap station. The center point of each trapping web contained 2 Sherman and 2 mesh traps at 90° angles to each other. The total number of traps in each sampling grid was 352. Each park was trapped 19 times (4 nights each time) from October 2002 through September 2005, approximately every 8 weeks. Traps were checked each morning. The sampling was specifically designed such that densities, diversities, and infection prevalence could be compared across space and time. The total trap effort (traps × nights) was 133,760 trap-nights. Sherman and mesh traps were baited with a mixture of peanut butter and rolled oats, Tomahawk traps were baited with cat food, and pitfall traps were not baited. To reduce deaths from hypothermia, we added polyfiber nesting material to Sherman and mesh traps when warranted by the weather.

All captured animals were treated as if they were infected with SNV, and standard precautionary methods were implemented (*25*). After point of capture was recorded, animals were transferred from traps to sealable plastic bags or, if too large, left in the trap and brought to the center of the web, where they were weighed and measured and examined for age, sex, reproductive status, scarring, or other notable characteristics. Retroorbital blood samples were collected by using heparinized microcapillary tubes and either placed in cryovials and frozen in liquid nitrogen or placed in serum separator tubes and refrigerated for no more than 1 week before testing. Infection prevalence was determined by ELISA (*26*). Infected deer mice were counted 1 time (time of first capture).

During the first 2 years of the study, to obtain tissue samples for a companion study, deer mice were euthanized in a chloroform chamber (*25*). The resulting specimens were tagged and stored at the Museum of Vertebrate Biology at Portland State University. All other animals captured were marked with ear tags and released at the point of capture. During the last year of the study, deer mice were also tagged and released. To determine whether removal affected subsequent capture rates within the same trapping period, the differences between the number of captures on the first and last day of the trapping period were calculated and averaged, then compared between removal and replacement sampling with the Welch 2-sample *t*–test. Because no significant differences were found between the first 2 years and the last year of the study ($t = 0.50$, $p = 0.63$, $df = 8$), data from all 3 years were analyzed together. This research was conducted under the auspices of federal, state, and city permits, and it complied with the American Society of Mammalogists' guidelines for animal care and use (*27*).

Species Diversity and Density

Deer mouse density was calculated by using the Distance program (*28*). Mammal species diversity was measured by using the Simpson diversity index (D_S) (*29*), which takes into account both richness (number of species) and evenness (number of individuals within each species) and ranges from 0 (least diversity) to 1 (maximal diversity). D_S further represents the probability of interspecies encounters (*30*). Pairwise comparisons of D_S values among parks was conducted by using the Student *t* test; differences of D_S were divided by the square root of their variances (*30*). To minimize the possibility of type 2 errors resulting from multiple comparisons, a statistically conservative Bonferroni correction was made ($\alpha = 0.05/10$ comparisons, or 0.005) (*31*). Deer mouse densities were compared pairwise by using the Welch 2-sample *t*-test. Logistic regression with binomial errors was initially used to assess the association between infection prevalence and deer mouse density and species diversity. However, the resulting models showed such extensive overdispersion that we considered logistic regression to be an unsuitable statistical method for these data (*32*). Accordingly, we used nonlinear regression analysis.

Analysis of Similarity

An analysis of similarity returns a statistic (R) based on a Bray–Curtis dissimilarity measure, which considers the difference of the mean ranks between and within groups. Most values fall between 0 and 1; 1 is the most dissimilar. Significance is assessed by comparing the observed value of R to the permutation distribution of R (*33*). Again, because of multiple comparisons, a Bonferroni correction was made such that $\alpha = 0.005$ (*31*). We then used stepwise (backward) logistic regression with binomial errors to assess the association between infection prevalence and vegetative characteristics.

Results

Although only 5 sites were examined, the intensity of the sampling yielded a total of 5,057 individuals from 21 species, resulting in a thorough species inventory over a gradient of diversity in small mammal ecological communities. Deer mice averaged 62% of all captures (Table 2) and were the dominant species at all sites. Mammal species diversity differed significantly among sites ($p<0.001$; Table 2), except sites 3 and 4 ($p = 0.1$). Densities varied spatially and temporally; all parks exhibited the highest densities during year 2 (Table 3). Interannual variances of densities were large due to seasonal differences in capture rates, such that no statistical differences in densities were found either within or among parks. Infection prevalence also varied, although it remained consistently low at 4 of the 5 sites.

Table 2. Small mammal capture data for 5 parks, Portland, Oregon, USA, October 2002–September 2005

Site no.	Total no. mammals captured	No. deer mice (*Peromyscus maniculatus*) captured	No. deer mice/total no. captured	No. species	Simpson diversity index, D_S
1	1,032	798	0.773	12	0.385
2	1,248	884	0.708	11	0.461
3	730	492	0.674	11	0.532
4	862	862	0.633	16	0.560
5	1,185	472	0.398	16	0.753

During year 1, infection prevalence was significantly higher at site 1 than at sites 2 and 3 ($p<0.001$) but not different than at sites 4 and 5 ($p = 0.20$ and 0.32, respectively). Site 1 was the only site where infection prevalence significantly increased between years 1 and 2 ($p = 0.005$); thus, infection prevalence at this site was significantly higher than at any of the other parks during year 2 ($p<0.001$). High infection prevalence was maintained at site 1 during year 3. Although the rate for site 2 increased significantly between years 2 and 3 ($p = 0.035$), prevalence remained significantly higher at site 1 than at any other park during year 3 ($p<0.01$).

Using nonlinear regression, we found a significant negative relationship between infection prevalence and mammal species diversity. Infection prevalence increased as diversity decreased, up to an inflection point where the rate of infection increased exponentially (Figure). No regression model was able to account for the association between infection prevalence and density of deer mice, either alone or with species diversity in the model.

A pairwise analysis of similarity was used to compare sites floristically; all parks differed significantly from each other ($p<0.001$). Stepwise backward logistic regression with binomial errors found no association between infection prevalence and any vegetative factors alone or in combination with other vegetative factors.

Discussion

Population densities fluctuated synchronously at all sites, yet infection prevalence increased significantly at only 1 site, which suggests that factors other than density alone are involved in disease transmission. If, as hypothesized, transmission were through aggressive encounters (*12*), SNV would spread most efficiently in an ecosystem composed solely of deer mice, where every encounter would be a potential disease-transmitting encounter. As more species, and more individuals within those species, are added to the community, the number of potential disease-transmitting encounters decreases because species other than deer mice are nonhost (not competent, or nonamplifying) species. This type of decreased intraspecies interaction has been termed "encounter reduction" (*34*) and would occur if increasing species diversity increases the number of competitors in an ecosystem, thereby increasing the amount of time a host species has to spend securing limited resources (food, nest sites), in turn decreasing the time spent on intraspecies encounters.

An increase in species diversity, in combination with an increase in the densities of individuals within those species, as we observed in this study, should also mean an increase in the number of predators of the rodent host species. It is reasonable to hypothesize that predators keep rodent numbers under control, in turn limiting pathogen spread both among rodents and into human populations, although it has been difficult to empirically support this hypothesis (*35*). Our results suggest that predators control infection prevalence not by controlling the density of host species but instead by an unrelated mechanism, possibly encounter reduction. When predators are present in the ecosystem, host species should spend more time in the nest, in hiding, or within the familiarity of their territory, all to avoid predation and all likely to decrease intraspecies encounters. This hypothesis is supported by the fact that capture rate—but not density—was highest at site 1 during year 2 relative to all other parks ($p<0.01$), which means that deer mice were moving about and encountering traps more often. We hypothesize that when predation and competition are decreased or absent, for this small mammal community at a Simpson diversity index ≈0.43, a zoonotic release of predatory and competitive controls appears to have occurred, in which SNV infection prevalence increased drastically. This

Table 3. Comparison of deer mouse (*Peromyscus maniculatus*) density and Sin Nombre virus infection prevalence, Portland, Oregon, USA, October 2002–September 2005

Site no.	Deer mouse density			Infection prevalence		
	Year 1	Year 2	Year 3	Year 1	Year 2	Year 3
1	6.78	22.38	8.76	0.049	0.141*	0.148
2	13.77	32.86	24.71	0.004†	0.011†	0.037*†
3	8.57	13.62	8.11	0.000†	0.013†	0.044†
4	15.92	23.34	7.30	0.015	0.004†	0.030†
5	7.74	23.43	7.80	0.021	0.012†	0.023†

*Significance between years at $\alpha = 0.05$ using a test of homogeneity of proportions with the Yates continuity correction.
†Significance between site 1 and other sites at $\alpha = 0.05$ with the Fisher exact test for count data.

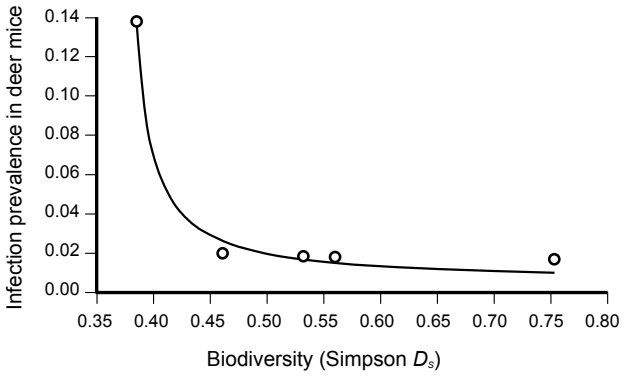

Figure. Results of the nonlinear regression analysis between species diversity (expressed as Simpson diversity index, D_s) and Sin Nombre virus prevalence among deer mice (*Peromyscus maniculatus*) at each of 5 parks in Portland, Oregon, USA. The best fit model was of the form Y = x / (ax + b), R^2 of 0.9994, p = 0.00001. The figure represents a summary of the results in that it shows the averages of all the seasons, in all years, in each park (indicated by circles). A regression using individual seasons and parks shows the same results.

hypothesis would account for the lack of differences in infection prevalence rates at sites 2–5; although the Simpson index for these sites varied significantly; the threshold for zoonotic release had not been breached at any of those sites. Above the threshold level, sites would maintain a low level of infection, or perhaps locally lose infection altogether. In this study, SNV infection prevalence was either so low during some seasons at some sites as to be virtually undetectable by traditional trapping techniques or ephemerally absent. In particular, SNV was undetected or absent most often at the most diverse site (no SNV was detected in 8 of 12 seasons at site 5, in 6–7 seasons at sites 2–4, and in 1 season at site 1). Our system differs from the Lyme disease system, which depends on a vector that is not host specific (black-legged tick) to transmit the disease. Here, in contrast, presence of nonhost species in the small mammal community will not directly affect the transmission of SNV; instead, the behavior of members of the natural host species will be affected, decreasing SNV transmission rates among competent hosts through encounter reduction. Increased diversity in both the Lyme disease and SNV systems appears to lead to decreased disease prevalence, although the mechanisms differ. Another difference between the 2 systems is the threshold relationship between species diversity and SNV prevalence, which suggests that the shape of the dilution curve may be mechanism dependent and is the reason we proposed the term "zoonotic release." Given that many hantaviruses are hosted by generalist rodent species (i.e., those able to exploit a broad variety of ecological resources) that dominate ecosystems as species diversity decreases (e.g., Laguna Negra virus in vesper mice [*Calomys* spp.]; Andes and Choclo viruses in colilargos [*Oligoryzomys* spp.]; and Calabazo virus in cane rats [*Zygodontomys* spp.]), this type of zoonotic release could be widespread throughout the host–virus system in the genus *Hantavirus*.

Host density should likewise be considered a factor in this phenomenon because density increased before infection prevalence increased. However, the result of the logistic regression between density and infection prevalence, although significant, was marked by considerable overdispersion, suggesting that this was the wrong model, and its significance was greatly overestimated (*32*). Additionally, at all parks deer mouse density increased but infection prevalence did not, clearly indicating that density is not the sole driver of infection prevalence in this system. A logistic regression with both density and mammal species diversity in the model showed similar overdispersion. Our results suggest that dependence on both density and frequency play a role in SNV transmission, which may be one of the reasons it has been so hard to determine their respective roles in the transmission of hantaviruses (*36*). More extensive studies should therefore be undertaken wherein species diversity, density, and frequency of encounters are carefully measured to determine their respective roles in disease transmission.

The finding that infection prevalence of a directly transmitted zoonosis may be inversely related to species diversity has implications for human health. The toll in illness and death from emerging zoonotic diseases is high, and outbreak investigations are costly (*37*). These investigations often fail to identify the source of a pathogen, let alone answer the question of why an outbreak occurred at a given time and place. If the host species or vector is found, eradication usually is neither possible nor desirable, particularly when the species are as ubiquitous as deer mice. Prophylaxis is difficult when transmission is airborne, as in hantaviruses, for which potentially everyone in a region is at risk. Ecosystem-level control may be the best way to protect the public from the increasing threat of many zoonotic diseases. Wildlife also are at risk for infection with novel pathogens, and the factors underlying wildlife disease emergence are similar to those in humans (*38*); a dilution effect may therefore help protect wildlife as well. For example, a study of West Nile virus suggested that increased bird species richness depressed the prevalence of the virus in ecosystems (*39*). Thus, wildlife could be protected in 2 ways: first, from dilution of diseases that are potentially harmful to them and second, from maintenance of healthy ecosystems.

Extension of a dilution effect to directly transmitted diseases has implications for conservation as well. Although protecting species diversity is a cause that would seem universal in its appeal, conservationists often are perceived as being overly biocentric and having little concern

for human welfare. In addition, many benefits derived from maintaining diverse ecosystems are difficult for the layperson to decode and seem far removed from daily life such that despite scientific research, unparalleled loss of species caused by anthropogenic factors continues at an unabated rate. Conservation likely will not succeed without the support of the general public, who in turn influence the environmental policies our society embraces. To gain support of the general public, tangible human benefits from conservation should outweigh the immediate—usually economic—gains of nonconservation land use (*40*). Linking human health to biodiversity could be just the benefit for gaining the public's support of conserving biodiverse ecosystems. Protection from disease is a tangible objective; it is easily understood and translated and it has direct benefits for all. As a consequence, extension of a dilution effect to directly transmitted diseases could have broad conservation implications by raising the public's concern about conservation in a manner that has yet to be emphasized.

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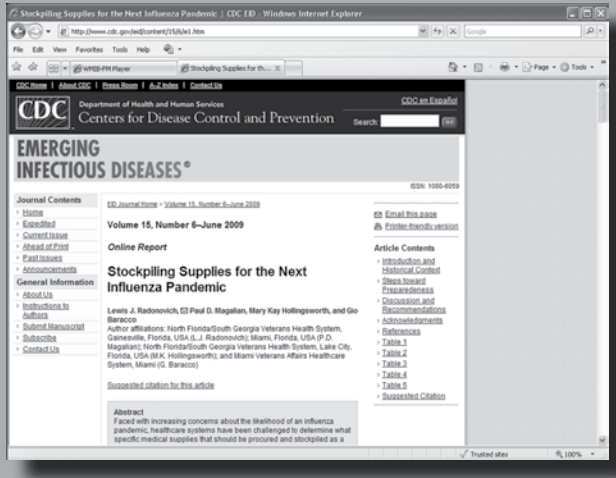

Spatial and Temporal Dynamics of Lymphocytic Choriomeningitis Virus in Wild Rodents, Northern Italy

Valentina Tagliapietra, Roberto Rosà, Heidi C. Hauffe, Juha Laakkonen, Liina Voutilainen, Olli Vapalahti, Antti Vaheri, Heikki Henttonen, and Annapaola Rizzoli

We determined the prevalence of infection with lymphocytic choriomeningitis virus (LCMV) among small mammals in northern Italy and analyzed long-term dynamics of LCMV in a rodent population in the province of Trento. LCMV is circulating among the most widespread and common wild rodent species in this area (*Apodemus flavicollis*, *Myodes glareolus*, and *Microtus arvalis*); overall prevalence is 6.8%. During 2000–2006, intensive monitoring of LCMV in a population of yellow-necked mice (*A. flavicollis*) showed a positive correlation between prevalence of infection and rodent density. At the individual level, weight and sex appeared to correlate with antibody prevalence, which suggests that horizontal transmission of LCMV occurs principally among heavier, older males and occurs during fighting. Isolation and genetic characterization of this virus will be the crucial next steps for a better understanding of its ecology.

Viral hemorrhagic fevers caused by arenaviruses pose serious human public health risks and cause devastating and often lethal disease. These diseases include Lassa hemorrhagic fever in West Africa, Junín hemorrhagic fever in Argentina, Machupo and Chapare hemorrhagic fevers in Bolivia, Guanarito hemorrhagic fever in Venezuela, and Sabià hemorrhagic fever in Brazil. In recent years, increased air travel between Africa and other continents led to the importation of cases of Lassa fever virus into the United States, Europe, Japan, and Canada and caused increasing concern about the potential of arenaviruses to trigger new emerging disease foci (*1*–*3*).

Lymphocytic choriomeningitis virus (LCMV) is a rodent-borne arenavirus (family *Arenaviridae*, genus *Arenavirus*) first reported in St. Louis, Missouri, USA, in 1934 (*4*). It is primarily associated with the house mouse (*Mus musculus*) (*5*); prevalence rates among this species range from 2.5% to 9.0% in the United States (*6*,*7*), 11.7% in Spain (*8*), 3.6% in Germany (*9*), and 7.0% in Japan (*10*).

LCMV commonly infects T cells in house mice, and these animals may act as carriers with long-term or lifelong viremia and viruria and negligible signs of acute disease (*5*,*11*). Several wild rodent species are seropositive for LCMV: *Mus spretus* (Algerian mouse), *Apodemus agrarius* (striped field mouse), *A. flavicollis* (yellow-necked mouse), *A. sylvaticus* (wood mouse), *A. mystacinus* (eastern broad-toothed field mouse), *Micromys minutus* (harvest mouse), *Microtus levis* (syn. *M. rossiaemeridionalis* [sibling vole]), *Chionomys roberti* (Robert's snow vole), *Myodes glareolus* (bank vole), and *Arvicola scherman* (montane water vole) (*8*,*12*–*15*). Hamsters and guinea pigs may also become infected after close contact with infected *M. musculus* mice and may also be asymptomatic (*16*). LCMV transmission in natural rodent hosts occurs vertically, horizontally, and during sexual intercourse. However, horizontal and vertical transmission may lead to different outcomes. Horizontal transmission may cause only transient viremia, and vertical transmission may cause chronic infection (*17*,*18*).

Humans become infected with LCMV by inadvertently inhaling aerosolized rodent excreta or secreta (*7*,*8*). Human-to-human transmission has not been reported, except for 1 case of vertical transmission from an infected mother to her fetus (*19*). LCMV-infected humans are generally

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asymptomatic or show mild influenza-like symptoms. However, LCMV infection can also lead to aseptic meningitis, meningoencephalitis, and congenital abnormalities (*20*). Immunocompromised persons are particularly susceptible to infection with LCMV; deaths caused by LCMV in organ transplant recipients have been reported (*21*).

Although LCMV is the only arenavirus reported in Europe (*12*), data on its incidence and epidemiologic features on this continent are insufficient. Only antibodies against LCMV in humans have been reported from Spain (1.7%) (*8*) and the Netherlands (2.9%) (*22*). In 2002, a preliminary survey of LCMV in the province of Trento in northern Italy showed an antibody prevalence of 5.6% for wild rodents (6.1% for *A. flavicollis*, 3.3% for *M. glareolus*, and 14.3% for *Microtus arvalis* [common vole]) and 2.5% for forestry workers (*14*). The occurrence of LCMV or LCMV-related viruses in several rodent species in Europe has led to the suggestion that LCMV could represent a complex of strains or closely related arenaviruses hosted by different rodent species (*12*,*14*).

We studied the distribution and prevalence of LCMV in small mammals throughout the Alps in northern Italy. Moreover, we analyzed the dynamics of LCMV in an intensive, long-term, capture-mark-recapture study of rodents in the province of Trentino in Italy. We also studied whether patterns of pathogen prevalence vary at the population level (density, season, time, space) or individual level (weight, sex, and breeding status).

Materials and Methods

Study Sites

Extensive sampling was conducted during 2002–2006 at 8 sites in northern Italy (Figure 1): 1 in Lombardy (province of Sondrio), 1 in Veneto (province of Belluno), and 6 in Trentino-Alto Adige (province of Trento). Intensive monitoring was conducted during 2000–2006 in Valle dei Laghi, province of Trento, in the northeastern Italian Alps (Dos Gaggio, Municipality of Cavedine, 50°56′15″N, 16°31′13.8″E) (Figure 1). This site is located on an isolated calcareous ridge (750–800 m above sea level), is dominated by broadleaf forest (*Fagus sylvaticus*, *Carpinus betulus*, *Fraxinus ornus*, *Corylus avellanae*), and includes plantations of larch (*Larix decidua*), spruce (*Picea abies*), and pine (*Pinus sylvestris, P. strobus*). Forest management includes coppices and coppices converted to high-stand forest. Small meadows (<1 hectare) are scattered throughout the woodland.

Rodent Monitoring

Extensive samples were obtained at the 8 sites. Of these sites, 6 were surveyed in 2002 during 1 session of 4 days and 3 nights, and 3 were surveyed in 2006 during 4 sessions of 4 days and 3 nights. Multicapture live traps (Ugglan Special Mouse Trap 2; Grahnab, Hillerstorp, Sweden) were used to capture animals. Live-trapped rodents were subsequently killed with isofluorane. During necropsy, samples from lungs, spleens, and kidneys were collected and stored at –80°C until analysis.

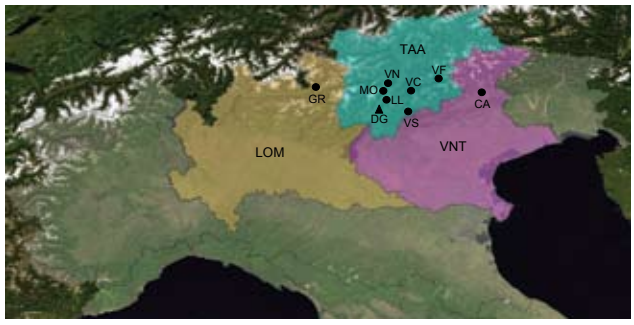

Figure 1. Study sites for trapping of rodents and isolation of lymphocytic choriomeningitis virus in Lombardy (LOM), Trentino-Alto Adige (TAA), and Veneto (VNT) in northern Italy, 2000–2006. GR, Grosotto/Mazzo; MO, Molveno; VN, Val Non; VC, Val Cembra; VF, Val Fiemme; LL, Laghi Lamar; DG, Dos Gaggio; VS, Val Sella; CA, Candaten. Circles indicate sites of extensive sampling and triangles indicate sites of intensive sampling. Background map: True marble by Unearthed Outdoors LLC (Madison, WI, USA) is licensed under a Creative Attribution 3.0 United States License (www.unearthedoutdoors.net/global_data/true_marble/download).

During 2000–2006 at Dos Gaggio, rodents were intensively live-trapped by using capture-mark-recapture techniques and Ugglan live traps (Grahnab). Trapping was conducted every 2 weeks for 2 consecutive nights from April through October and occasionally from November through March on 8 × 8 grids (64 traps) with a 15-m distance between traps. Nine grids were used during 2000–2002, and 4 grids were used during 2003–2006. At first capture, a passive-induced transponder (ID 100; Trovan, Hessle, UK) was implanted subcutaneously into each animal. Species, sex, breeding condition, and weight were recorded.

Because small rodent populations are seasonally and multiannually heterogeneous, individual rodents were categorized into the following functional groups (*23–25*): juveniles, ≤1 month of age, gray pelage [fur], and weight <15 g); subadults, weight >15 g and not in breeding condition (undescended testes or imperforate vagina); and adults, weight >15 g and in breeding condition (descended testes or perforate vagina, visible nipples, or visibly pregnant). At the end of the breeding season, some postbreeding adults may by definition appear to be subadults. To ensure that postbreeding animals were not included in the subadult category, animals that were defined as adults once during the season were considered adults for all statistical analyses. Blood samples were collected once per 2-week trapping session from the suborbital venous plexus by using a mi-

crohematocrit capillary tube (length 75 mm, diameter 1.15 mm). Blood samples were centrifuged and serum samples were stored at –20°C until analysis.

Rodent Densities

Population density of yellow-necked mice (*A. flavicollis*) at intensively studied sites was estimated by using the Jolly-Seber mark–recapture model (*26*). Population density at extensive sites was determined by calculating the small mammal abundance index (SAI) according to the equation SAI = (SC × 100) / (T × N), in which SC is the number of rodents captured, T is the number of traps, and N is the number of nights).

Antibody Assays

All serum samples were tested for immunoglobulin G against LCMV by using an indirect immunofluorescent antibody assay as described (*12,27*). The LCMV strain used in this assay was obtained from the Swedish Institute for Infectious Disease Control (Stockholm, Sweden). Animals that were positive or weakly positive for LCMV and animals that were negative after showing a positive result at a previous trapping session were retested when possible. LCMV-positive animals were assumed to have a chronic infection.

Statistical Analyses

To assess spatial differences in seroprevalence of LCMV at many sites, we used a generalized linear model with a binomial error and S-PLUS version 7.0 software (TIBCO Software Inc., Palo Alto, CA, USA). The binary response variable was the presence or absence of virus antibodies in mouse serum samples, and explanatory variables were province; trapping site; rodent weight, sex, and breeding status by species; rodent abundance index; and trapping year.

For the intensive dataset for Dos Gaggio, only data for *A. flavicollis* mice were analyzed (at population and individual levels) because other rodents of other species were rarely trapped at this site and none were infected with LCMV. To assess whether antibodies against LCMV in *A. flavicollis* mice were affected by any host or population characteristics, we used generalized linear mixed models (GLMMs) with a penalized quasilikelihood algorithm and binomial and S-PLUS version 7.0 software. In this analysis, the presence of antibodies against LCMV was the response variable. For population analysis, rodent density and trapping month and year were the explanatory variables. For individual analysis, sex, breeding status, and weight were selected to identify the model that best explained variance in the presence of virus antibodies.

To overcome autocorrelations caused by multiple trapping of the same rodent, the unique transponder code of each animal was entered into GLMMs as a random effect. Variance explained by each explanatory factor and levels of significance were calculated by using a stepwise backward deletion test (*28*).

Results

During 99,464 trap nights (9,864 in extensive monitoring and 89,600 in long-term intensive monitoring), 2,342 rodents in 5 species (*A. agrarius*, *A. flavicollis*, *A. sylvaticus*, *M. glareolus*, and *M. arvalis* were trapped. *A. flavicollis* and *M. glareolus* were the most frequently trapped species (87.6% and 5.7%, respectively). A total of 3,215 serum samples (2,732 at Dos Gaggio and 483 in the extensive sampling) were analyzed.

Extensive Sampling

The overall prevalence of LCMV was 8.3% (40/483) (Table). Antibodies were detected in all species except *A. agrarius* and *A. sylvaticus*. The highest prevalence was in *M. arvalis* voles (20%), although sample size for this species was low (n = 5). For the more abundant and ubiquitous species (*A. flavicollis* and *M. glareolus*); prevalence was 8.9% and 7.4%, respectively.

The seroprevalence rate was highest in Val Sella (28.6%; 2/7), but the number of samples was low. The province of Belluno in the region of Veneto had a preva-

Table. Prevalence of lymphocytic choriomeningitis virus in 5 rodent species at extensive trapping sites, northern Italy, 2002 and 2006*

Region	Province	Site	Rodent species					Total no. rodents	Seroprevalence, % (no. positive/ no. tested)
			Apodemus agrarius	*A. flavicollis*	*A. sylvaticus*	*Myodes glareolus*	*Microtus arvalis*		
VNT	BL	Candaten	2	44	1	5	0	52	3.8 (2/52)
LOM	SO	Grosotto/Mazzo	0	49	0	32	0	81	9.9 (8/81)
TAA	TN	Val Cembra	0	97	4	26	5	132	8.3 (11/132)
TAA	TN	Val Fiemme	0	11	0	7	0	18	5.5 (1/18)
TAA	TN	Laghi Lamar	0	63	0	34	0	97	3.1 (3/97)
TAA	TN	Molveno	0	30	0	1	0	31	12.9 (4/31)
TAA	TN	Val Non	0	35	0	30	0	65	13.8 (9/65)
TAA	TN	Val Sella	0	7	0	0	0	7	28.6 (2/7)
Total			2 (0)	336 (8.9)	5 (0)	135 (7.4)	5 (20)	483 (8.3)	

*VNT, Veneto; BL, Belluno; LOM, Lombardy; SO, Sondrio; TAA, Trentino-Alto Adige; TN, Trento.

lence of 3.8%. Seroprevalence rates in Sondrio (region of Lombardy) and Trento (region of Trentino-Alto Adige) were 9.9% and 8.8%, respectively. None of the explanatory variables of the extensive dataset (province, trapping site, species, sex, weight, breeding status, rodent abundance index, trapping year) affected the presence of antibodies against LCMV in the rodent species sampled, except in *A. flavicollis* mice.

Temporal Dynamics of LCMV in *A. flavicollis* Mice

During 2000–2006 (89,600 trap nights) at Dos Gaggio, 1,717 yellow-necked mice were trapped and 7,523 recaptures were reported. This population shows a multiannual and seasonal variation; the population density begins to increase in the spring (end of February through March), marking the beginning of the reproductive period; reaches a peak in mid-summer (end of July through the end of August); and is followed by a decrease during the winter months. In 2001 and 2005, a second peak occurred in autumn. The years with the highest density were 2000 (10.78 animals/hectare) and 2005 (17.03 animals/hectare), both of which followed a year of extensive seed production (masting) of beech trees at this site, which apparently favored rodent survival and prolonged the mating season into the winter months. The population structure (Figure 2) showed a large number of juveniles captured in the spring of high-density years that followed years of extensive seed production, which confirmed that mouse reproduction extended into the intervening winter.

A total of 205 (7.5%) of 2,732 serum samples from *A. flavicollis* mice contained antibodies against LCMV. The prevalence per year ranged from 0.97% to 14.1%; rodent populations had the highest seroprevalence in 2002 (14.14%, 57/403), followed by 2005 (9.88%, 113/1144), 2006 (5.36%, 17/317), 2003 (5.04%, 7/139), 2004 (2.73%, 5/183), 2000 (1.18%, 4/389), and 2001 (0.97%, 2/207).

The model that best explains the difference in LCMV seroprevalence at the population level includes trapping year and rodent density. Specifically, model coefficients showed a significant difference in prevalence among trapping years ($F_{6,1579} = 15.13$, $p<0.001$) and a positive correlation with rodent density ($F_{1,1579} = 68.36$, $p<0.001$) (Figures 3, 4).

At the individual level, seroprevalence showed a positive correlation with weight ($F_{1,1545} = 240.04$, $p<0.001$). Juvenile and subadult mice were significantly less likely to be infected than adults ($F_{2,1545} = 101.1$, $p<0.001$). LCMV seroprevalence was not influenced by sex ($F_{1,1545} = 0.04$, $p = 0.82$), but sex and weight produced a significant interaction; stronger positive effect of weight on LCMV seroprevalence was stronger for male rodents than for female rodents ($F_{1,1545} = 4.57$, $p = 0.032$).

Discussion

The prevalence and transmission rates of rodent-borne viruses in host populations vary in time and space and among host–virus systems. Improving our understanding of the causes of these variations will lead to a better understanding of changes in disease risk to humans. Our study highlights the endemicity of LCMV (or LCMV-related viruses) in small rodents in northern Italy and complements reports of LCMV seroprevalence in humans in Italy (*14*).

Our results show that LCMV or LCMV-related viruses are circulating among the most widespread and common wild rodent species other than the typical reservoir (*M. musculus*). These species are *Microtus arvalis* (prevalence 20%), *A. flavicollis* (8.9%) and *M. glareolus* (7.4%) (Table). Only *A. agrarius* and *A. sylvaticus* mice were not positive for LCMV antibodies. These results confirm those of previous studies (*14*,*15*), which detected antibodies against LCMV in rodent species of the subfamily Arvicolinae. Additional arenaviruses may be present in Europe.

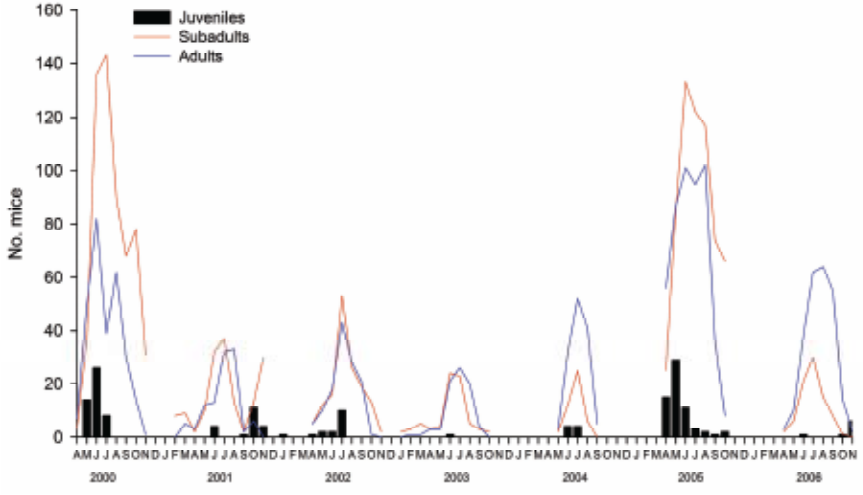

Figure 2. Population structure of *Apodemus flavicollis* in Dos Gaggio region of Trentino Alto-Adige, northern Italy, 2000–2006. Juveniles, ≤1 month of age, gray pelage (fur), and weight ≤15 g); subadults, weight >15 g and not in breeding condition (undescended testes or imperforate vagina); adults, weight >15 g and in breeding condition (descended testes or perforate vagina, visible nipples, or visibly pregnant). Gaps in the plots indicate that no trapping was conducted during these periods.

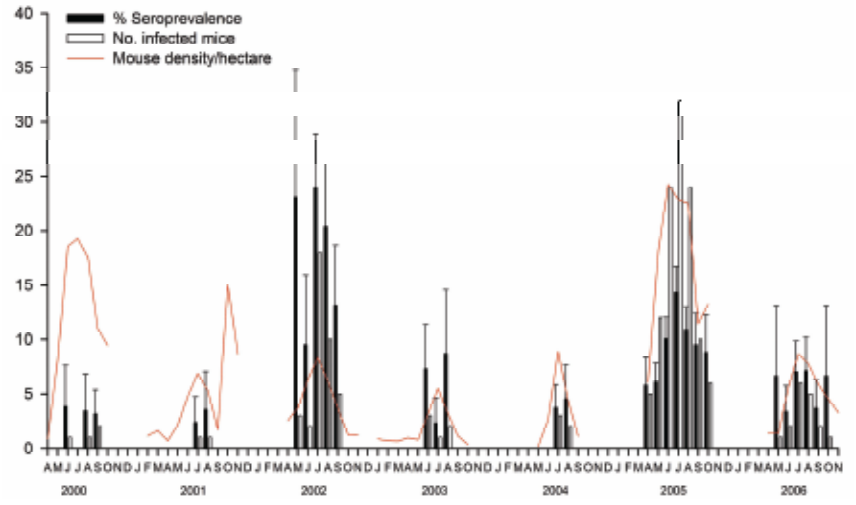

Figure 3. Correlation between dynamics of arenavirus seroprevalence, number of infected rodents, and density of *Apodemus flavicollis* in Dos Gaggio region of Trentino Alto-Adige, northern Italy, 2000–2006. Error bars indicate standard errors. Gaps in the plots indicate that no trapping was conducted during these periods.

Overall prevalence of LCMV among rodents in our study, including results from extensive and intensive sampling sites, was 6.8%, which is comparable to that reported in the study of Kallio-Kokko et al. (*14*) (5.6%). All of our sites had rodents positive for LCMV; prevalences were higher in the provinces of the central Alps (8.8% in Trento and 9.9% in Sondrio) than in Belluno in the eastern Alps (3.8%). Because all trapping grids were set in similar habitats, the density and diversity of rodent species were comparable between provinces. LCMV appears to be less common in the eastern Alps than in the central Alps. Further investigation is needed to determine the reasons for this difference.

The long-term intensive trapping system used at Dos Gaggio provided a unique opportunity to document the dynamics of LCMV in a rodent community dominated by *A. flavicollis* mice. Use of GLMMs provided a powerful tool for overcoming nonindependence of data resulting from repeat samples taken from the same rodent. Our analysis showed that mean annual population density showed a correlation with prevalence of infection in *A. flavicollis* mice (Figure 3). This result is consistent with what is known about the behavior of this species. Increases in density also increase overlap between neighboring home ranges, the number of contacts, and conflict between rodents and thus increase the potential for virus transmission (*29*,*30*). Two exceptions are evident in the 2 years of high density of rodents (2000 and 2005). In 2000, LCMV prevalence was particularly low, but low prevalence may have been caused by the low number of samples analyzed (20% of the total). The low prevalence finding in 2005 could have been caused by the large proportion of juveniles captured, which, as our data indicate, tend to be LCMV negative and would lower overall prevalence.

Sporadic production of mast-producing trees, such as beech, is an important environmental factor that affects the dynamics of many forest rodents in temperate Europe. Mast-driven outbreaks in bank voles (*M. glareolus*) in Belgium have led to outbreaks of nephropathia epidemica, a mild form of hemorrhagic fever with renal syndrome caused by Puumala hantavirus (*31*). Our results imply that masting also affects the dynamics of the yellow-necked mouse and, consequently, the multiannual dynamics of LCMV in this host species. Because antibodies against LCMV in Europe have been found in *A. flavicollis* mice from Italy (this study), Turkey (*13*), and Finland (*12*), masting-induced rodent dynamics may also affect the human incidence of LCMV in temperate areas of Europe. We are currently testing this hypothesis.

Our analysis also indicates clear seasonal variation; the number of infected mice increased as mouse density increased during the breeding season (Figure 4), although month did not appear to affect seroprevalence. This finding suggests that transmission between mice is not primarily be-

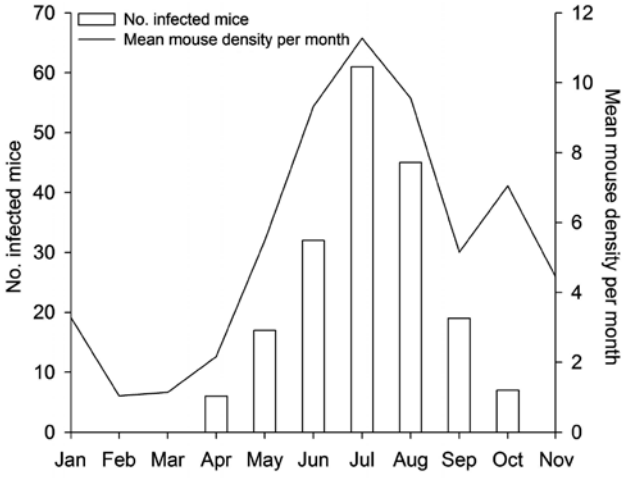

Figure 4. Monthly number of lymphocytic choriomeningitis virus–positive animals and mean rodent density per month (pooled data), northern Italy, 2000–2006.

tween animals of different sex. Our results were consistent with those of Laakkonen et al. (*12*), who showed that sex of the mouse does not affect LCMV prevalence. However, our results suggest that weight and sex interact and show a correlation with antibody prevalence in host populations, as does age, so that heavier, older males are most likely to be LCMV positive. This result suggests horizontal transmission of LCMV by a mechanism that involves mainly males, such as infection by bite wounds inflicted during fighting (*32*–*35*). This hypothesis is supported by previous reports that male mice have a greater home range than females, and their home ranges overlap more than those of more territorial females (*29*,*36*,*37*). The fact that juveniles and subadults are less frequently infected than adults suggests that maturation and behavioral changes also play a role in virus transmission. Furthermore, although our results indicate that intraspecies transmission and maintenance of LCMV in *A. flavicollis* mice are dependent on social and spacing behavior in this species, other factors, such as genetic and physiologic variation at the individual, population or species level, could affect transmission.

Finally, for technical reasons, LCMV obtained from wild rodent species in Europe has not yet been isolated or sequenced. However, genetic characterization of arenaviruses is obviously crucial to the understanding of the ecology and epidemiology of LCMV and is one of our immediate goals.

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etymologia

Borrelia

[bə-rel′e-ə]

Named after French bacteriologist Amedée Borrel (1867–1936) in 1907, *Borrelia* is a genus of bacteria, family Spirochaetaceae, made up of gram-negative, irregularly coiled helical cells that surround a central fibrillar substance. These organisms cause tick-borne and louse-borne relapsing fever in humans and animals. For example, *B. hermsii*, transmitted by *Ornithodoros hermsi* ticks, causes relapsing fever in the Western United States, and *B. recurrentis* causes louse-borne relapsing fever worldwide. Another member of the genus, *B. burgdorferi*, isolated from patients with arthritis-like symptoms by Willy Burgdorfer and Alan G. Barbour in 1982, is the etiologic agent of Lyme disease.

Although Borrel did not work extensively with spirochetes, he published several articles on *Spirillum* (now *Borrelia*) *gallinarum*. He is also known for searching for an infectious cause of cancer and for proposing that this agent could be a virus.

Source: Dorland's illustrated medical dictionary, 31st edition. Philadelphia: Saunders; 2007; Wright DJM. Borrel's accidental legacy. Clin Microbiol Infect Dis. 2009;15:397–9.

Tick-borne Relapsing Fever and *Borrelia hermsii*, Los Angeles County, California, USA

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The primary cause of tick-borne relapsing fever in western North America is *Borrelia hermsii*, a rodent-associated spirochete transmitted by the fast-feeding soft tick *Ornithodoros hermsii*. We describe a patient who had an illness consistent with relapsing fever after exposure in the mountains near Los Angeles, California, USA. The patient's convalescent-phase serum was seropositive for *B. hermsii* but negative for several other vector-borne bacterial pathogens. Investigations at the exposure site showed the presence of *O. hermsi* ticks infected with *B. hermsii* and the presence of rodents that were seropositive for the spirochete. We determined that this tick-borne disease is endemic to the San Gabriel Mountains near the greater Los Angeles metropolitan area.

Tick-borne relapsing fever was first observed in California, USA, in 1921 when 2 persons were infected in a cabin in Nevada County, north of Lake Tahoe (*1*). Another 251 cases of relapsing fever were reported in this state through 1941. Most persons who became ill had exposures at high elevations in various mountain locations (*2*). During this time, tick-borne relapsing fever was found to be endemic near Big Bear Lake in the San Bernardino Mountains, San Bernardino County, in southern California (*2*,*3*). The first human case in the Big Bear Lake area was reported in 1930 (*3*); for the next 12 years, more people became ill with relapsing fever there than in any other single area in the state (*2*).

The initial human cases of relapsing fever in California preceded identification of the vector, which was discovered to be a previously unidentified tick subsequently named *Ornithodoros hermsi* (*4*,*5*). Naturally infected ticks that were collected near Big Bear Lake and Lake Tahoe transmitted spirochetes in the laboratory when the ticks fed on monkeys, mice, and a human volunteer; this transmission showed the role of *O. hermsi* ticks as vectors (*4*,*6*). In 1942, Davis named the *O. hermsi* tick-associated spirochete *Spirochaeta hermsi* (*7*), now recognized as *Borrelia hermsii* (*8*).

Early investigations of relapsing fever associated with *O. hermsi* ticks at Big Bear Lake and Lake Tahoe were seminal for defining the epidemiologic parameters that maintain the enzootic foci in these locations and in other appropriate habitats throughout western North America (*9*). However, since the early field and laboratory studies performed in the 1930s, little work has been conducted to further elucidate the distribution of *B. hermsii* in southern California. In 1989, a total of 6 persons were infected sequentially while staying at different times in the same cabin near Big Bear Lake (*10*), and a year later, 2 persons were diagnosed with relapsing fever when they were hospitalized in Santa Monica, California, after a weekend visit to Big Bear Lake (*11*). During these outbreaks, ticks were not collected and spirochetes were not isolated from the patients' blood. The most recent review of relapsing fever that included California reported 95 cases for 1978–1998 (*12*). During this 21-year period, 16 (17%) persons were infected in San Bernardino County, but no specific locations were provided.

Information regarding the presence of an enzootic focus of relapsing fever in Los Angeles County, California, is

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scant and old. A review published in 2002 showed no cases of relapsing fever originating in Los Angeles County during the 20th century (*13*), and a monograph on California ticks had no record of *O. hermsi* ticks being found there (*14*). In 1942, Beck reported 2 cases for 1936–1940 and noted that *O. hermsi* ticks were found in a "nest in garage" at an elevation of 2,134 m (7,000 feet) (*2*). However, no additional information was provided, and we are unaware of any reports that substantiate the presence of human relapsing fever, *O. hermsi* ticks, or *B. hermsii* spirochetes near the Los Angeles metropolitan area. The California State and Los Angeles County health statistics units had no reports of relapsing fever having ever originated in Los Angeles County.

On September 7, 2006, a 51-year-old man cleaned a room associated with a 100-year-old solar telescope at Mt. Wilson Observatory, located at 1,737 m (5,700 feet) elevation in the San Gabriel Mountains, Los Angeles County, in southern California. Numerous rodent nests were present in old cardboard boxes in the room, and the floor was littered with rodent feces, acorn husks, and other debris suggesting many years of rodent occupation. On September 9, the patient carried several cardboard boxes containing rodent nests, and some of the material transferred to his clothing. Later that evening, he noticed 2 "insect bites," 1 on each leg, just above the sock line. The bites were asymptomatic erythematous macular lesions that enlarged from 2 mm to 6 mm in diameter over the next few days and faded after a week.

On the evening of September 17, the patient experienced a sudden onset of weakness, fever to 38.9°C, shaking chills, and joint and muscle pain; nausea and vomiting developed the next day. On September 20, as the illness persisted, he sought help at a clinic and was prescribed antiemetics, but showed no improvement. On September 21, as the illness worsened, he sought help at a hospital emergency department. Physical examination indicated dehydration and fever (38.9°C). A complete blood count showed mild thrombocytopenia and increased granulocyte count (Table 1). He was treated with ketorolac, metoclopramide, and intravenous saline for possible viral illness and was released. The patient improved over the next several days, but his illness relapsed on September 27, when he had increased weakness, arthralgias and myalgias, fever, shaking chills, and renewed nausea and vomiting. The patient's illness peaked on September 28 and improved on September 29, but his condition again relapsed on October 1. The patient returned to the emergency department and was hospitalized. Physical examination indicated a temperature of 39.1°C. He was treated with intravenous fluids, antiemetics, and piperacillin/tazobactam. Complete blood counts showed leukocytosis, left shift, and thrombocytopenia (Table 1). Routine blood cultures, which do not support borrelia growth, were negative. He improved a few hours after receiving antimicrobial drug treatment but was mildly hypotensive for a day. The antimicrobial drug was discontinued on October 3, and the patient was again released. Night sweats continued for 2 days after discharge, and he felt generalized weakness for the next several days. He returned to work full time on October 16.

Methods

Site Investigation

Because of a suspicion that the patient had contracted tick-borne relapsing fever, the exposure site was investigated for ticks. A member of our research team (L.S.W.) designed a novel tick trap in which a white terry cloth towel was wrapped around small blocks of dry ice, which emits CO_2 and attracts ticks (*15*). Each trap was taped to the end

Table 1. Laboratory test results for 4 dates during illness of patient presumably ill with tick-borne relapsing fever, Los Angeles County, California, USA, 2006

Test	Sep 21	Oct 1	Oct 2	Oct 3	Reference range
Hematocrit, %	43.3	42.3	33.7	33.3	39–55
Erythrocyte count, $\times 10^{-6}$/mm^3	4.6	4.6	3.6	3.6	4.3–5.9
Platelet count/mm^3	114	145	127	162	130–450
Leukocyte count/mm^3	8.8	11.5	12.8	9.5	4.8–10.8
Neutrophils, %	83	86	64	58	50–70
Band cells, %		5	11	5	0–4
Urea nitrogen, mg/dL	16	10	11	9	6–20
Creatinine, mg/dL	1.2	1.1	1.1	1.0	0.5–1.2
Aspartate aminotransferase, U/L		32	25	29	10–42
Alanine aminotransferase, U/L		68	52	55	10–60
Total bilirubin, mg/dL		2.2	1.5	1.2	0–1.5
Alkaline phosphatase, U/L		206	143	140	42–121
Sodium, mg/dL	134	133	138	140	135–145
Potassium, mg/dL	3.1	3.6	4.5	4.3	3.6–5.0
Glucose, mg/dL	133	112	119	107	70–110
Albumin, g/dL		2.7	2.1	2	3.2–5.5

of a 1-m stick and placed in recesses of the room where the patient was potentially exposed to ticks; the traps were left overnight and checked the next morning. During several nights in late October 2006, the traps collected 6 ticks, which were sent to the Rocky Mountain Laboratories, where they were identified as *O. hermsi* nymphs and tested for spirochete infection.

During the nights of June 12–14, 2007, and May 15, 2008, multiple dry ice traps were set again at the observatory in several buildings with abundant signs of rodent activity, including the room that provided the previous ticks. One *O. hermsi* tick was collected in 2007, and 2 nymphs were collected in 2008, all from the previously infested room where the patient had presumably been bitten.

On July 16 and October 23–24, 2008, Tomahawk (Tomahawk Live Trap Co., Tomahawk, WI, USA) and Sherman (H.B. Sherman Traps, Tallahassee, FL, USA) live traps were set on the observatory grounds to capture rodents for serologic testing for antibodies against *B. hermsii*. Whole blood samples were collected from 22 rodents, and the serum samples were tested by immunoblot with whole cell lysates of *B. hermsii* and purified recombinant glycerophosphodiester phosphodiesterase Q (GlpQ), an immunodominant protein in relapsing fever spirochetes but absent from Lyme disease spirochetes (*16*).

DNA Amplification by PCR and Sequence Analysis of Spirochetes

DNA was extracted from the 6 ticks collected in October 2006 and examined by PCR and DNA sequence analysis of the 16S rRNA, flagellar B protein (*flaB*), gyrase B (*gyrB*), *glpQ*, and variable tick protein (*vtp*) genes and the noncoding intergenic spacer (IGS) locus of *B. hermsii* by using methods described (*17*–*19*).

Tick Feeding and Spirochete Isolation

The 3 *O. hermsi* nymphs collected on June 14, 2007, and May 15, 2008, each fed on a laboratory mouse (*Mus musculus*). On days 3–9 after feeding, the mice had ruffed hair, and blood collected from the tail vein showed numerous spirochetes per field in a wet preparation viewed by dark-field microscopy at 400×. Each mouse was anesthetized, bled by intracardiac puncture, and euthanized, and 0.2 mL of the blood was passed intraperitoneally into another mouse. The mice were moribund 2 days later and had extremely high spirochetemias. The mice were euthanized immediately after intracardiac puncture, and the blood samples were inoculated into Barbour-Stonner-Kelly (BSK)–H medium (Sigma-Aldrich Inc., St. Louis, MO, USA) with 12% rabbit serum for spirochete isolation. Thin smears of the infected blood were made on glass microscope slides and fixed with methanol to test spirochete reactivity with monoclonal antibody H9724, which is specific for all *Borrelia* spp. (*20*), and monoclonal antibody H9826, which is specific for *B. hermsii* (*21*). DNA was purified from the spirochete cultures for PCR and DNA sequence analysis, and aliquots of the cultures were frozen at –80°C. Our work with the animals was reviewed and approved by the Rocky Mountain Laboratories Animal Care and Use Committee (protocol no. 2006–10).

Serologic Tests

A convalescent blood sample was drawn from the patient on March 17, 2008, after he signed the informed consent under a protocol approved by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Institutional Review Board. The serum was tested by the indirect immunofluorescent antibody (IFA) assay with spirochetes fixed on glass microscope slides, including *B. hermsii* originating from the exposure site, *B. hermsii* DAH originating from eastern Washington, and the Lyme disease spirochete *Borrelia burgdorferi* B31. The serum was also tested with fixed antigen preparations of *Rickettsia rickettsii*, *Coxiella burnetii*, and *Yersinia pestis*, the agents of Rocky Mountain spotted fever, Q fever, and plague, respectively. The serum was tested at 8 serial 2-fold dilutions from 1:16 to 1:2,048, and goat anti-human immunoglobulin G conjugated with fluorescein isothiocyanate (1:100) (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD, USA) was used as the secondary antibody. IFA titers were determined by viewing with a Nikon (Tokyo, Japan) Eclipse E800 epifluorescence microscope at 600× and oil immersion. Serum was also examined at 1:100 dilution by immunoblot with whole cell lysates of the same spirochete panel and with recombinant GlpQ purified by nickel chromatography, as described (*16*,*22*).

Results

The patient's clinical history and the ecologic setting of his exposure led us to suspect that he had contracted tick-borne relapsing fever, and we initiated a search for the tick vector. Six *O. hermsi* tick nymphs were collected with the dry ice traps and pooled for molecular analysis to determine presence of spirochete infection. DNA was purified from the pooled ticks, and PCR produced amplicons of the appropriate size for all loci examined. DNA sequences were determined for the 16S rRNA, *flaB*, *gyrB*, *glpQ*, *vtp*, and IGS loci, which identified the Mt. Wilson spirochete (designated MTW-1) as *B. hermsii*, belonging to genomic group II (GG II) (*18*) (GenBank accession nos. EU194839, EU194840, EU194841, EU194842, EU203147, and EU203149). The 16S rRNA, *glpQ*, and IGS sequences were unique from all other sequences for these loci in our *B. hermsii* isolate collection. These unique sequences showed that the PCR products resulted from infected ticks, not from laboratory contamination. The *vtp* sequence was

identical to other Type 6 sequences found in genomic group I (GG I) spirochetes.

The 3 nymphal *O. hermsi* ticks collected on June 14, 2007, and May 15, 2008, each fed on mice (Figure 1) and transmitted spirochetes that produced high spirochetemias (Figure 2). These spirochetes bound both monoclonal antibodies, which identified the spirochetes as *B. hermsii* (data not shown). Spirochetes grew to high cell density in BSK-H medium, and DNA was purified from the second–passage, 100-mL cultures (*23*) and designated MTW-2, MTW-3, and MTW-4. DNA samples purified from the cultures were subjected to PCR, and DNA sequences of the amplicons were determined for the same 6 loci (GenBank accession nos. EU194843, EU194845, EU194846, EU194847, EU203148, and EU203150). All sequences determined for each locus were identical for the 3 isolates. Sequences for the 16S rRNA, *flaB*, and *gyrB* genes and for the IGS locus were identical to those sequences for MTW-1. This similarity identified MTW-2, MTW-3, and MTW-4 as *B. hermsii* belonging to GG II. The *glpQ* sequences for MTW-1 and for MTW-2, MTW-3, and MTW-4 contained 2 synonymous base differences (99.8% identity). However, the *vtp* DNA sequence for MTW-2, MTW-3, and MTW-4 were identical and unique from all other *vtp* sequences in the database (*18*), although the sequences showed similarities with other Vtp type 5 sequences with 94.6% identity (*18*). The *vtp* sequences in MTW-1 and MTW-2 represented different Vtp types (type 6 and 5, respectively) and shared only 78.5% DNA and 69.3% amino acid identities.

Setting the traps resulted in capturing 22 small mammals from 4 species: 14 California ground squirrels (*Spermophilus beecheyi*), 2 Merriam chipmunks (*Tamias merriami*), 5 brush mice (*Peromyscus boylii*), and 1 California mouse (*Peromyscus californicus*). Immunoblot analysis of serum samples from the 22 animals showed that 2 of the brush mice were strongly reactive with antibody reactivities equal to those of the positive control sample (Figure 3). The other 20 samples were negative. The positive serum samples contained antibodies that bound to ≥9 proteins in the *B. hermsii* whole-cell lysates and to the recombinant purified GlpQ. The 2 seropositive mice were captured in traps set against the exterior walls of the building in which the patient was presumed to have been infected.

The patient's convalescent-phase serum sample was seropositive for relapsing fever with the highest IFA titer (2,048) to *B. hermsii* MTW-2 originating from Mt. Wilson and with the lowest titers (512 and 128) to *B. hermsii* DAH from eastern Washington and Lyme disease spirochete *B. burgdorferi*, respectively (Table 2). IFA titers were negative with the other bacterial antigens (Table 2). Immunoblots were also positive for antibodies against GlpQ, and the serum samples had the strongest reactivity to the Mt. Wilson spirochete (data not shown).

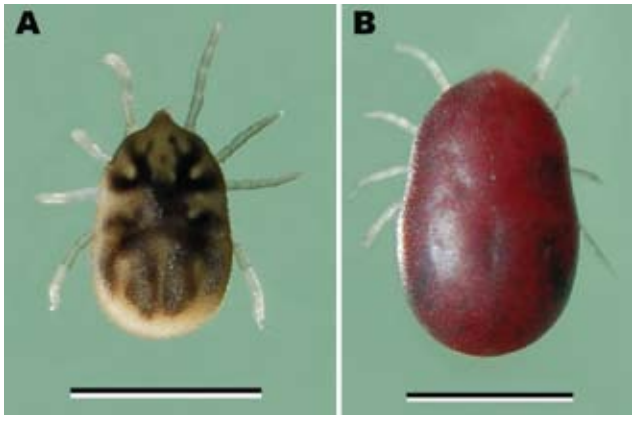

Figure 1. *Ornithodoros hermsi* nymphal tick from Mt. Wilson, California, USA. Panel A shows the nymph before its infective blood meal; panel B shows it after feeding. Scale bars = 2 mm.

Discussion

The diagnosis of relapsing fever was not considered during the patient's illness, and his blood was not examined for spirochetes during his acute-phase episodes. Thus, we were unable to isolate spirochetes from his blood or obtain an acute-phase serum sample to compare with the convalescent-phase sample. However, we conclude that the patient had contracted relapsing fever on the basis of several factors: his clinical history and exposure consistent for this disease, his recovery with antimicrobial drug treatment, the high IFA titer to *B. hermsii*, the positive immunoblot, the presence of *O. hermsi* ticks in the room of exposure, the presence of *B. hermsii* in the tick pool, and the isolation of spirochetes that originated from 3 naturally infected ticks. Additionally, the patient lived on Mt. Wilson and had not traveled recently to other known locations where this disease is endemic. Cumulatively, these findings demonstrate an enzootic focus of tick-borne relapsing fever in Los

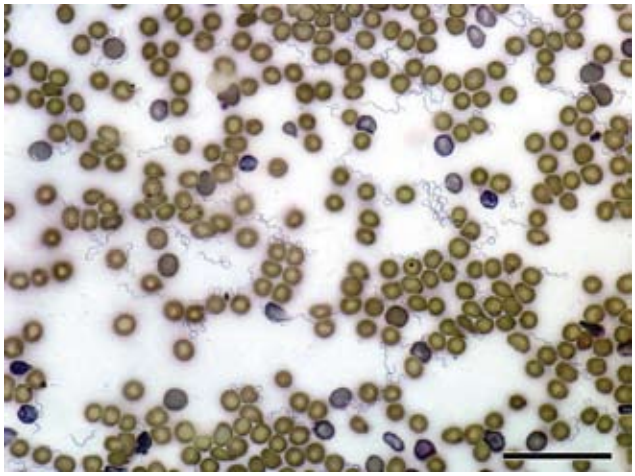

Figure 2. *Borrelia hermsii* MTW-2 in mouse blood (Wright-Giemsa stain) viewed at 600× oil immersion. Scale bar = 40 μm.

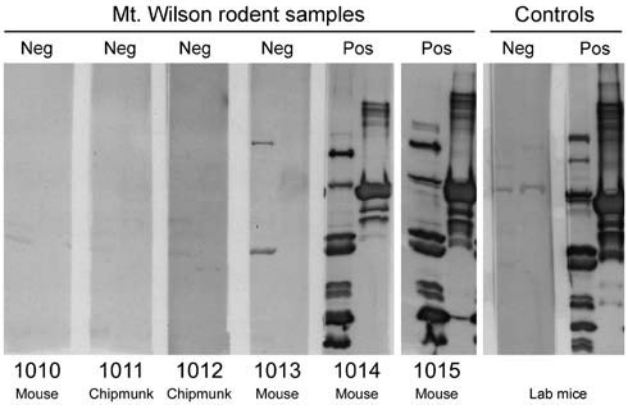

Figure 3. Immunoblot analysis of serum samples from brush mice (*Peromyscus boylii*) and Merriam chipmunks (*Tamias merriami*) captured at Mt. Wilson Observatory in California, USA. Each sample was tested at a dilution of 1:100 with a whole-cell lysate of *Borrelia hermsii* MTW-2 isolated from Mt. Wilson (left lane of each membrane) and purified recombinant GlpQ (right lane of each membrane). Results and animal numbers are presented above and below each panel, respectively. Neg, negative; pos, positive.

Angeles County at a location only 10 km (6.5 miles) from downtown Pasadena.

A few vertebrate animals potentially involved with this focus were investigated. Douglas tree squirrels (*Tamiasciurus douglasii*), which are important hosts for *O. hermsi* ticks and *B. hermsii* spirochetes, inhabit the Sierra Nevada Mountains to the north but are absent from the San Gabriel and San Bernardino Mountains (*9*,*24*,*25*). Other sciurids, including the California ground squirrels (*S. beecheyi*), Western gray squirrels (*Sciurus griseus*), and Merriam chipmunks (*T. merriami*), are abundant on the observatory grounds. Several species of mice (*Peromyscus* spp.) are also found at the observatory. In 1947, Longanecker collected 90 *O. hermsi* ticks from an active deer mouse nest near Big Bear Lake (*26*). Many larval and nymphal ticks had recently fed, and some specimens were infected with spirochetes. Our serologic results suggest that brush mice (*P. boylii*) are involved in the enzootic focus on Mt. Wilson.

Western bluebirds (*Sialia mexicana*) are also abundant around the observatory and nest in tree hole cavities. *O. hermsi* ticks have been found in bluebird nests in the Sierra Nevada Mountains and in British Columbia, Canada (*26*,*27*); these birds may also serve as hosts for the ticks in this site. Further work is needed to determine the role that these and other mammals and birds may play in maintaining the ticks and spirochetes in the mountains of southern California.

Table 2. Patient convalescent-phase IFA assay titers to *Borrelia hermsii* and other vector-borne bacterial pathogens, Los Angeles County, California, USA, 2006*

Species	IFA titer
B. hermsii MTW-2	2,048
B. hermsii DAH	512
B. burgdorferi B31	128
Rickettsia rickettsii	<16
Coxiella burnetii	<16
Yersinia pestis	<16

*IFA, indirect immunofluorescent antibody.

The investigation provided several results concerning the distribution and genetic diversity of *B. hermsii*. First, spirochetes from all ticks from Mt. Wilson belonged to GGII, extending south by ≈900 km (560 miles), the known geographic distribution of *B. hermsii* in this genomic group (*19*). Therefore, spirochetes in both genomic groups are likely to occur throughout the north-to-south range of these organisms in far western North America. Second, GGII spirochetes were found in naturally infected *O. hermsi* ticks; all GGII isolates examined previously came from blood of clinically ill patients (*19*). Third, the Mt. Wilson spirochetes MTW-1 and MTW-2 had identical or nearly identical DNA sequences at all loci examined except for the variable tick protein gene (*vtp*). The antigenically diverse Vtp sequences occurring in ticks in the same location may allow spirochetes of a specific Vtp type to reinfect previously infected hosts that are possibly immune to spirochetes of another Vtp type (*18*).

Our study found an enzootic focus of the relapsing fever spirochete *B. hermsii* and its tick vector *O. hermsi* on Mt. Wilson near the Los Angeles metropolitan area. The patient in our study probably contracted relapsing fever there, although he was not tested for this infection during his illness. During the third acute-phase episode, he was hospitalized; hospitalization occurs frequently among patients if this disease is not diagnosed early. A retrospective analysis of relapsing fever cases in California and Washington for 1995–2005 showed that 46% and 80% of cases in each state, respectively, required hospitalization (*28*). Therefore, we emphasize that physicians and other healthcare providers should consider relapsing fever as a possible diagnosis when patients seek treatment for an acute, febrile, recurrent disease after being exposed in the mountains of southern California and other regions where relapsing fever is endemic. We also recommend collection of blood samples during acute episodes for subsequent analysis and possible retrospective clinical testing and diagnosis.

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Risk Factors for Human Infection with Puumala Virus, Southwestern Germany

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Puumala virus, which causes nephropathia epidemica (NE), is the most prevalent hantavirus in Germany; bank voles serve as the main reservoir. During 2001–2007, most NE cases reported from Germany occurred in the southwestern state of Baden-Württemberg. We investigated the influence of bank vole habitats (beech forest, seed plants), vole food supply (beechnut mast), climate factors (winter and spring temperatures), and human population density on spatial and temporal occurrence of NE cases in Baden-Württemberg. Using Poisson-regression analyses, we found that all these factors influenced disease incidence. Furthermore, an independent trend of increasing incidence predicted that incidence will nearly double each year. The regression model explained 75% of the annual variation in NE incidence. The results suggest that environmental drivers lead to increasing incidence of NE infections in the southern part or even other parts of Germany.

Hantaviruses (family *Bunyaviridae*) are the etiologic agents of 2 distinct clinical syndromes: hemorrhagic fever with renal syndrome and hantavirus pulmonary syndrome (*1*,*2*). The former occurs in Asia and Europe; the latter, in the Americas (North, Central, and South) (3). Of the ≈30 hantaviruses described, approximately half are of clinical relevance (*4*–*6*). In Germany, the most common hantaviral disease is nephropathia epidemica (NE), which is associated with Puumala virus (PUUV) infection (*7*,*8*). The primary rodent reservoir for PUUV is the bank vole (*Myodes glareolus,* formerly *Clethrionomys glareolus*), which in Europe extends south from Scandinavia to Italy and Spain (*9*).

NE is considered a mild form of hemorrhagic fever with renal syndrome; mortality rate is <1%. After an incubation period of 2–4 weeks, disease onset is abrupt; major signs and symptoms are fever, headache, back pain, abdominal pain, and other gastrointestinal involvement. Occasionally, acute renal failure develops and the patient may require hemodialysis.

Transmission of hantaviruses from rodents to humans is believed to occur through inhalation of aerosols contaminated by virus shed in excreta, saliva, and urine of infected animals (*10*). Human-to-human transmission of hantaviruses is rare, although investigations in Argentina implicated Andes virus with this type of infection (*11*).

One hypothesis suggests that the risk for human infection with hantaviruses increases with the population size of the reservoir host species, which can be driven to high levels in response to events that enhance host survival, promote early breeding, and increase the food supply (*12*). Such events may result from climatic perturbations, such as the El Niño Southern Oscillation, which increases precipitation and results in unusually mild winters. It has been hypothesized that *Peromyscus maniculatus* rodents, the reservoir host for Sin Nombre virus (SNV), increased as a result of the El Niño Southern Oscillation (*13*). In Europe, researchers recently demonstrated a positive relationship between tree seed production, milder climate, and NE incidence (*14*). The availability of suitable habitat for rodents is also a key factor to consider when assessing the risk for hantavirus transmission.

Although environmental factors influence the availability and quality of suitable rodent habitat and resources, risk for hantaviral disease transmission to humans also

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depends on human proximity, behavior, and land-use patterns. Furthermore, peridomestic exposure might increase as rodent reservoirs increase and rodents disperse to buildings. Therefore, investigations assessing risk for hantaviral infection must evaluate factors influencing the reservoir host population, the human population at risk, and potential factors driving their interaction.

Since NE became a notifiable disease in Germany in 2001, most cases have been reported from the state of Baden-Württemberg, in southwestern Germany (*15*), where the number of cases varied from a minimum of 17 in 2006 to a maximum of 1,077 in 2007. Our objective was to investigate the association between NE incidence in southwestern Germany and environmental factors that potentially influence the PUUV reservoir abundance and, hence, the risk of acquiring NE. We used statistical modeling to assess the influence of vole habitat (presence and quality), annual variation in vole food source, variation in climatic conditions, and the human population at risk on the annual NE incidence during a 7-year period. Specifically, we investigated whether NE incidence is positively associated with availability of suitable rodent habitat and supply of food, human population density, and a rise in winter or spring temperatures above long-term averages.

Materials and Methods

Incidence of NE

The annual total of patients with symptomatic, laboratory-confirmed cases of NE reported from the state of Baden-Württemberg was provided by the German surveillance system for infectious diseases, which covers all 44 districts of Baden-Württemberg, for 2001–2007. Laboratory diagnosis was based either on detection of viral RNA by reverse transcription–PCR or on detection of immunoglobulin (Ig) M or a marked rise of antihantavirus IgG (*15*). Details of the German notification system are provided by Faensen et al. (*16*), and detailed information about the incidence of NE in Germany can be obtained from Piechotowski et al. (*15*). We excluded from analysis those patients who reported recent travel. We obtained human population density by district from the Statistical Office in Baden-Württemberg (*17*). Because the population of Baden-Württemberg in the respective districts did not change substantially during 2001–2007, we used census data for 2006 to calculate incidence rates (no. cases/100,000 inhabitants) for each study year.

Bank Vole Factors, Habitat, and Beechnut Mast

Factors considered to favor bank vole habitat were obtained from the Forest Research Institute Baden-Württemberg, Department of Biometry and Information Science, from forest inventories conducted during 2001–2002 (*18*). We assessed percentage of land cover for 5 covariates that have been indicated as preferred habitat for bank voles in Europe (*19*,*20*): beech forests, seed plants, bilberries, dwarf shrubs, and blackberries. For oak forests, also a preferred habitat, no data were available. Data for beech forest cover were provided as hectares per district and converted to proportion coverage per district area. For each of the 4 remaining habitat areas by district, data were provided as area variables in 3 categories of coverage: rare (1%–10%), frequent (>10%–50%), and common (>50%). To estimate the availability of the respective habitat in each district, we calculated a weighted sum of the 3 coverage areas by using 0.01, 0.1, and 0.5 as weights for the rare, frequent, and common areas, respectively, and then converted each sum into percentage coverage per district area. For each district, the 5 habitat variables were considered constant during the study period.

We obtained data on annual mast production of beechnuts by district, starting and ending 1 year earlier than the study (2000–2006). Data came from the Ministry for Agriculture Baden-Württemberg and the Public Forest Administration Baden-Württemberg, for which foresters conduct annual counts of beechnuts under beech trees (plot counts) (*21*). The beechnut mast in the preceding year was used as a potential determinant of the NE incidence because beechnut supply in the fall may influence winter vole survival and, consequently, vole population the following year. Beechnut mast data were stratified into 3 classes: good/excellent crop if 40%–100% of trees produced mast, medium if 10%–39%, and poor if 0%–9%.

Climate Factors

Deviations of the monthly temperature for 2000–2007 were referenced against the perennial average for 1961–1990, provided by the German Meteorological Service (*22*). The station that geographically best represented a respective district was selected from the network of 576 meteorology stations covering Germany. Only the temperature deviations of the winter and early spring months (December–March) were included because mild winters and springs were hypothesized to enhance survival rates of rodents and produce food resources earlier. For each year and district we modeled mean values of temperature deviations for winter (December of the preceding year and January of the same year as NE incidence data) and spring (February and March of the same year).

Statistical Modeling

We modeled associations between potential risk factors and incidence rates of NE during 2001–2007 by multivariate Poisson regression, using the SAS program GENMOD (version 9.1, SAS Institute Inc., Cary, NC, USA). In Poisson regression, we set the logarithm of the expected

annual incidence per district and year equal to a linear term of potential determinants: habitat variables, climate factors, human population density, and year of investigation. The number of NE cases per year and district were approximately Poisson distributed. To allow for overdispersion, we introduced a scale parameter in the regression modeling. To account for nonlinearity, the variable "year of investigation" was represented in the regression model by 6 dichotomous variables. All other independent variables were considered continuous and were scaled by units as per 5% increase in district-area coverage by beech forest and seed plants, per increase in human population density of 500 inhabitants per square kilometer, per 1°C change in winter and spring temperature above the long-term average, and per unit step of beechnut mast (good/excellent, medium, poor). The covariates in the Poisson regression model were examined for 2-way interactions, but none could be confirmed.

The criterion for inclusion of a determinant in the final regression model was set at a significance level of $p \leq 0.05$. For a measure of association between a determinant and NE incidence, the risk ratio (RR) was calculated by using the respective estimated regression parameter of the Poisson regression model and, therefore, adjusted for all other determinants included in the regression model. All estimates of RR were complemented by a 95% confidence interval (CI) and p value. The pseudo-R-squared (R^2) was provided as a measure of overall goodness-of-fit of the regression model.

Results

A total of 1,540 NE cases were reported from the study area during 2001–2007 and were included in the analysis. The median values of NE incidence in all districts varied from 0 (in 2002, 2003, and 2006) to 2.28 in 2007 (Table 1); the lowest maximum incidence rate was 1.16 in 2006, and the highest maximum incidence rate was 90.19 in 2007. Mapping of the cumulative district NE incidence in Baden-Württemberg indicated that the districts reporting the highest incidence rate for cases clustered within the southeastern Swabian Alb region during 2001–2007 (Figure 1, panel B).

Time-dependent Factors

The annual percentage of statewide beechnut mast varied by year and district from a crop failure (0%–9% of optimum mast) in 2005 to good/excellent (40%–100% of optimum mast) mast in 2001 and 2006 (Table 1). Winter temperatures exceeded long-term averages in 2001, 2003, 2004, and 2007. The maximum winter temperature deviation occurred in 2007; median deviation was +3.5°C and maximum was +4.5°C. Winter temperatures were below the long-term averages in 2002, 2005, and 2006 (range of deviation –0.5°C to –1.5°C). Mean spring temperature exceeded the long-term average for all years except 2005 and 2006 (Table 1).

Time-independent Factors

Maximum beech forest cover within a district (17.7%) was found in the region of the Swabian Alb (Table 2; Figure 1, panel C); lowest beech forest cover was found in the eastern and middle regions, as well as in districts containing the major cities of Baden-Württemberg. Maximum seed plant cover (11.3%) was found in the southerly and centrally located districts of Baden-Württemberg; the lowest cover (1.3%) was associated with the northerly districts and in the districts containing the major cities. The median values of the land cover variables of dwarf shrubs, bilberry, and blackberry were <2%; values of ≈10% were restricted to a few districts (Table 2). As the percentages of land cover in bilberry and dwarf shrubs were closely correlated across districts ($r \approx 1$), only 1 of these variables (bilberry) was included in the regression modeling. Human population density ranged from 104 inhabitants/km^2 in rural districts to 2,864 inhabitants/km^2 in the state capital of Stuttgart (Table 2; Figure 1, panel B). Most districts (80%) contained <1,000 inhabitants/km^2.

Table 1. Nephropathia epidemica in Baden-Württemberg, Germany, by year, 2001–2007*

Year	No. cases†	Incidence/ 100,000 population			Temperature, °C						Beechnut crop¶		
					Winter‡			Spring§					
		Min	Med	Max	Min	Med	Max	Min	Med	Max	Min	Med	Max
2001	37	0.0	0.25	4.5	2.0	2.5	3.5	2.0	2.5	2.5	0	0	1
2002	140	0.0	0.0	11.18	−0.5	0.5	0.0	3.0	3.5	3.5	1	2	2
2003	55	0.0	0.0	3.33	0.5	1.0	1.5	−0.5	0.0	1.0	1	1	2
2004	109	0.0	0.27	6.01	0.5	0.5	1.5	0.0	1.0	1.5	1	1	2
2005	105	0.0	0.70	4.07	0.0	−0.5	1.0	−1.5	−1.0	−0.5	1	1	2
2006	17	0.0	0.0	1.16	−2.0	−1.5	−1.0	−2.0	−1.5	−1.0	0	0	0
2007	1,077	0.0	2.28	90.19	3.0	2.5	3.0	2.0	2.5	3.0	2	2	2

*Min, minimum; med, median; max, maximum.
†Total no. cases for all districts in Baden-Württemberg.
‡Temperature deviation from the long-term average for December of the previous year and January of the actual year.
§Temperature deviation from the long-term average for February and March.
¶Beechnut crop of the preceding year in 3 categories: 0, poor crop (0%–9% of a mast year); 1, medium crop (10%–39% of a mast year); 2, good/excellent crop (40%–100% of a mast year).

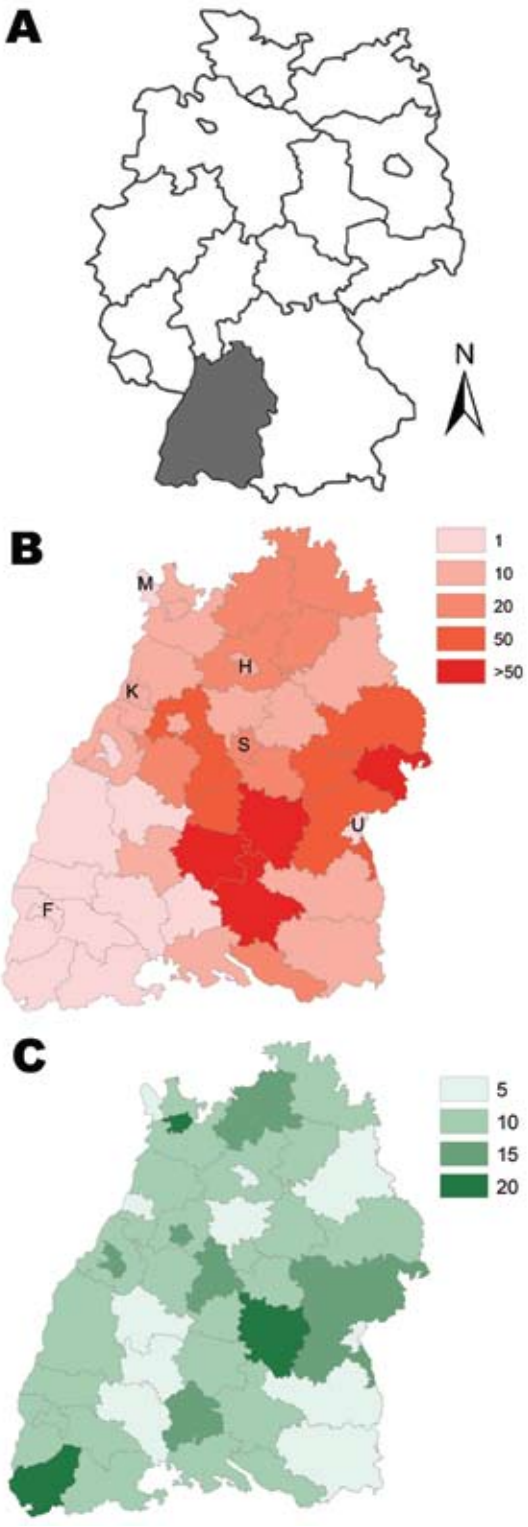

Figure 1. A) Map of Germany showing location of Baden-Württemberg region (gray shading). B) Cumulative incidence (per 100,000 population) of nephropathia epidemica, Baden-Württemberg, Germany, 2001–2007. Letters indicate major cities: F, Freiburg; H, Heilbronn; K, Karlsruhe; M, Mannheim; S, Stuttgart; U, Ulm. C) Percentage cover of beech forest.

Poisson Regression Analysis

For the final Poisson regression model, only the blackberry and bilberry variables did not pass the inclusion criterion of a positive association with the annual incidence of NE during 2001–2007 and a significance level of $p \leq 0.05$ (Table 3; Figure 2). The final model explained 75% of the variation of NE incidence ($R^2 = 0.75$).

The variables showing annual variation, specifically beechnut mast and spring temperature above the long-term mean, were strong independent predictors of NE. The occurrence of a good/excellent beechnut mast in the previous year increased risk for NE (RR 2.86, 95% CI 1.81–4.50) relative to medium crop (Table 3). An increase of 1°C in spring (February/March) above the long-term average resulted in RR of 4.49 (95% CI 2.86–7.06). The influence of winter (December/January) temperature was less strong, but it was still a significant factor in NE incidence; RR 1.70 (95% CI 1.11–2.61).

The association between NE incidence and year of investigation indicated that incidence increased exponentially during 2002–2006, when all other risk factors were controlled for and 2001 was used as a reference (Figure 3). However, this trend was not apparent in 2007. In 2007, extreme winter and spring temperatures relative to the long-term average, coupled with the best beechnut mast observed during the entire study interval, potentially masked or overwhelmed the temporal trend toward increasing NE incidence in the regression modeling. To check stability of the observed time trend, we repeated the regression analysis with the investigation year 2007 excluded. Again, a highly significant time trend, which indicated a doubling of the NE incidence per year (RR 2.02, 95% CI 1.43–2.83), was observed. Habitat and climate factors as well as human population density had somewhat lower, but still highly significant, estimates of RR (data not shown). When the estimated 2001–2006 regression model was used for prediction, the incidence in 2007 was considerably underestimated. This result underlines the importance of the time-dependent habitat and climate factors for the PUUV reservoir.

As notification of NE cases became a requirement in Germany in 2001, underreporting could have occurred in the first year of investigation (2001). However, exclusion of 2001 in the regression analysis did not change the significance and magnitude of the associations of the included risk factors with the NE incidence (data not shown).

Among the time-invariant determinants, 2 of the land-cover parameters—percentage of beech forest (Figure 1, panel C) and seed plant cover—exhibited a major effect. For each 5% increase in coverage per district, risk for NE approximately doubled (beech forest, RR 1.94, 95% CI 1.69–2.22; seed plants, RR 2.80, 95% CI 2.31–3.40; Table 3). A unit increase in human population density of 500 inhabitants/km^2 (about the median population density of the

Table 2. Bank vole habitat and human population density per district, Baden-Württemberg, Germany, 2001–2007

Density	Habitat cover, % total district area					Human population density/km^2 district area
	Beech forest	Seed plants	Dwarf shrubs	Bilberry	Blackberry	
Minimum	1.6	1.3	0.0	0.0	0.2	104
Median	8.1	3.9	0.2	0.2	1.8	323
Maximum	17.7	11.3	10.6	10.3	6.2	2,864

44 districts) increased incidence of NE by ≈12% (RR 1.12, 95% CI 1.01–1.23).

Discussion

The analysis of NE in the state of Baden-Württemberg during 2001–2007 clearly indicated a strong association of variables reflecting preferred bank vole habitat and abundance of a major food resource (beechnut mast), in addition to relative mild spring and winter temperatures, with the spatial and temporal incidence of PUUV infection. Furthermore, human population density was a weak but statistically significant determinant of NE incidence. The results also indicate that risk of acquiring NE increased over the 7-year study period, possibly forecasting a trend of increasing incidence of PUUV infection in southern Germany. The estimated Poisson regression model accounted for 75% of the spatial and temporal variation in NE incidence during 2001−2007.

The direct influence of mild winter and spring temperatures on NE incidence cannot be interpreted independently from other annual fluctuations, such as the quality of beechnut mast. As an example, winter and spring temperatures during 2000–2001 were as much as +3.5°C and +2.5°C above the long-term average, but the beech mast in 2000 was one of the lowest reported, and NE incidence was low in 2001 (median 0.25). When winter and spring temperatures above long-term average were coincident with a good/excellent beech mast, as in 2006–2007, incidence of reported NE in 2007 was the highest recorded (median 2.28) (Table 1).

After mild winter and spring conditions, and when supplied with a rich food resource from the previous fall, rodent populations likely reach higher densities through a combination of increased overwinter survival rate and earlier onset of breeding. Increased rodent abundance or density could increase risk for human contact with a PUUV-infected rodent and, thus, increase risk for NE. In the Great Basin desert area of the western United States, higher population densities of the rodent reservoir host for SNV and, consequently, increased incidence of hantavirus pulmonary syndrome have also been hypothesized to be driven by weather anomalies, which result in increased food sources after milder winters with increased rainfall (*23*,*24*). Mild winter was also hypothesized as a factor leading to an outbreak of PUUV infection 2007 in northern Sweden (*25*). However, in northern European locations where NE is endemic, different factors may contribute or drive the risk for human infection by PUUV. In Sweden, most human NE cases occur in late autumn or early winter and are believed to occur when rodents seeking to avoid harsh winter conditions move into human dwellings for shelter (*26*). In Germany, most cases occur in early summer (*15*), possibly when recreational and occupational activities bring people into bank voles' environments. Outdoor activities may also contribute to human peridomestic exposure to PUUV. A case–control study conducted in 2007 in Baden-Württemberg demonstrated that visiting or cleaning human shelters in the forest, among other factors, increased risk of acquiring the disease (*27*).

The influence of time-independent determinants also highlights patterns influencing risk for NE. The strong associations of beech forest and seed plant cover with NE incidence support the hypothesis that indices of preferred bank vole habitat, where bank vole populations reach

Table 3. Influence of determinants on incidence of nephropathia epidemica, Baden-Württemberg, Germany, 2001–2007*

Determinant	Risk ratio†	95% Confidence interval	p value‡
Supply of beechnut§	2.86	1.81–4.50	<0.0001
Cover of beech forest¶	1.94	1.69–2.22	<0.0001
Cover of seed plant¶	2.80	2.31–3.40	<0.0001
Winter temperature deviation#	1.70	1.11–2.61	0.0156
Spring temperature deviation#	4.49	2.86–7.06	<0.0001
Human population density**	1.12	1.01–1.23	0.0265
Year of investigation††	NA	NA	<0.0001

*Multivariate Poisson regression analysis; NA, not applicable.
†Mutually adjusted.
‡Likelihood ratio test
§Good/excellent crop year referenced to a medium crop year.
¶Unit = 5%.
#Unit = 1°C.
**Unit = 500/km^2.
††Dichotomized (Figure 2).

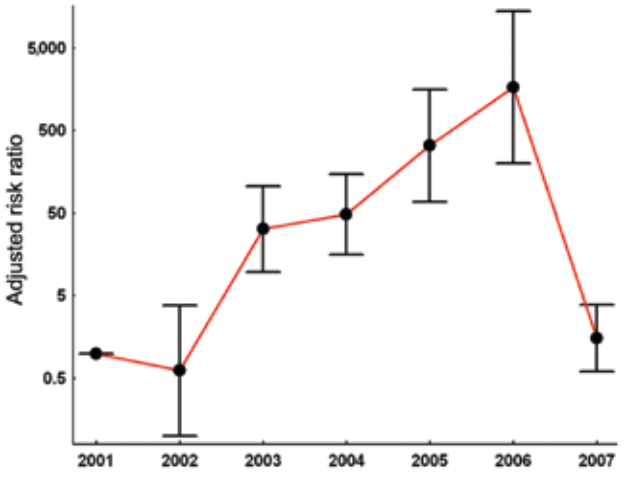

Figure 2. Influence of the year of investigation on the incidence of nephropathia epidemica, indicated by adjusted risk ratios estimated by Poisson regression analysis. 2001 is reference year. Error bars indicate 95% confidence intervals. For controlling covariates, see Table 3.

their highest density (*28*,*29*), are associated with elevated levels of PUUV transmission. The area with the highest percentages of beech forest and seed plant coincided with the Swabian Alb region, to which NE is highly endemic and the highest incidence rate (90.19) has been reported. In contrast, NE is rarely reported from the Black Forest region, which contains the highest elevations of Baden-Württemberg (as high as 1,500 m) and has a primary land cover of coniferous forest, a habitat not preferred by bank voles (*28*,*29*).

Other studies have also demonstrated a strong link between habitat indices for a rodent species serving as a reservoir host for a hantavirus and increasing risk for human disease. In Sweden, increasing abundance of bank voles and increasing numbers of PUUV-infected voles were associated with an environment composed of old-growth moist forests (*30*). In the Great Basin desert of the United States, deciduous or mixed forest, grasslands, and pinyon–juniper woodlands are associated with varying risk for hantavirus pulmonary syndrome (*24*,*31*).

The potential for human interaction with bank voles being a risk factor for NE incidence was suggested by the positive and significant association of NE incidence with human population density. Density served as a surrogate measure of actual human–rodent contact, which occurs when animals enter buildings or when humans participate in outdoor activities. Other surveillance-based studies of a zoonotic virus have linked human abundance with risk for disease exposure (*32*).

Additional biologic and environmental factors may be associated with the risk for hantavirus transmission to humans or may influence rodent abundance. Annual variation in precipitation has been suggested (*23*,*24*). Geo-ecologic variables, such as elevation, slope, or geology and soil type, have been linked to the risk for hantavirus infection in the US Great Basin (*24*). Indeed, the geologic substrate provides some measure of the dominant land-cover classes of vegetation. Beech trees prefer karst, with the soil types rendzina and cambisol, which is mostly found on the Swabian Alb, where most NE cases occurred. Other studies have shown that increased predators decrease populations of bank voles (*33*,*34*). An effect of predators on community composition and species abundance has been suggested for other vector-borne diseases, including hantaviruses (*35*,*36*).

Precise information on where NE was acquired was unavailable and therefore precluded our ability to analyze these site-specific factors. Therefore, a case–control study was initiated to provide a more detailed analyses of site-specific risk factors and to collect better information on presumed locations where transmission of PUUV occurred (*27*).

Surveillance data are subject to bias (*37*). Because the designation of NE as a notifiable disease in Germany is relatively recent, spatial and temporal modeling of surveillance data enhance the usefulness of these data by predicting disease trends and potentially assessing the quality of disease reporting (*37*).

Our results suggest that global climate anomalies, or the increasing trend toward warmer annual temperatures, could have a considerable effect on NE in Germany. Many regions in the world appear to be at increased risk for outbreaks of vector-borne and zoonotic diseases such as Rift Valley fever, West Nile fever (*38*), and NE (*15*,*16*,*25*,*27*). The effect of vector-borne and zoonotic diseases is dynamic and strongly linked to environmental drivers in addition to changes in human demographics and behavior. The current dispersion of NE in Germany and the increasing incidence, especially in the state of Baden-Württemberg, pose a risk to public health and require monitoring. Because no vaccine against NE is available and the potential costs of medical care associated with severe disease can be high, public health recommendations for reducing the risk for PUUV infection should be further promoted and evaluated, as has occurred in the United States (*39*). Analyses such as ours can help focus future studies and enhance surveillance efforts and evaluation of prevention measures by predicting where humans are at greatest risk for NE.

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Invasions by Eurasian Avian Influenza Virus H6 Genes and Replacement of the Virus' North American Clade

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The spread of highly pathogenic avian influenza virus (AIV) (H5N1) underlines the potential for global AIV movement through birds. The phylogenies of AIV genes from avian hosts usually separate into Eurasian and North American clades, reflecting limited bird migration between the hemispheres. However, mounting evidence that some H6 sequences from North America cluster with Eurasian subtype H6 sequences calls the strict hemispheric divide into question. We conducted a comprehensive phylogenetic analysis of the extent and timing of cross-hemisphere movements by the H6 gene. Results suggested that Eurasian H6 subtype has invaded North America several times, with the first invasions occurring 10 years before the first detection of invading isolates. The members of the North American clade decreased from 100% in the 1980s to 20% in the 2000s among H6 isolates from North America. Unraveling the reasons for this large-scale gene movement between hemispheres might identify drivers of global AIV circulation.

The transboundary expansion of highly pathogenic avian influenza virus (AIV) (H5N1) in 2005 increased the interest in global AIV spread by wild birds (*1*,*2*). Although the degree to which wild birds contribute to the large-scale spread of highly pathogenic subtype H5N1 influenza viruses has been much debated (*3*–*5*), consensus exists that natural barriers limit the movement of the virus between the Eastern and Western Hemispheres (*2*,*6*). To date, the spread of highly pathogenic AIV subtype H5N1 has been confined to Eurasia and Africa. Understanding the potential for genetic interchange of AIV between hemispheres is critical to limiting spread and mitigating the effects of AIV on human and animal health (*1*). Phylogenetic trees of most AIV gene segments and subtypes demonstrate a clear separation between North American and Eurasian clades, reflecting limited virus movement through bird migration between the hemispheres (*7*). However, some subtype H6 AIV strains isolated in North America were recently found to cluster in the Eurasian clade (*8*–*10*), which calls the belief in a strict hemispheric divide into question.

Subtype H6 (hereafter called H6) AIV infection occurs frequently in wild and domestic birds in Asia, America, and Africa (*11*–*16*). Although H6 is a subtype with low pathogenicity, outbreaks of H6 AIV in domestic birds have had a serious impact on the poultry industry in California (*17*). In addition, recent reports indicate a subtype H6N1 virus, A/teal/HK/W312/97, shared a common source of 6 of 8 gene fragments with a subtype H5N1 virus, A/Hong Kong/156/97 (H5N1) (*18*–*20*); the latter caused an outbreak among chickens, with sporadic human cases and deaths in Hong Kong during 1997 (*21*–*23*).

Given the importance of cross-hemisphere AIV movement for the United States, evidence for cross-hemisphere H6 AIV movement deserves closer examination. In this study, we investigated whether previous indications of cross-hemisphere movement of H6 AIV were part of a larger viral movement pattern. We constructed a phylogenetic tree using all currently available full-length H6 sequences and analyzed the spatial and temporal distribution of all sequences that showed a mismatch between genetic and geographic proximity to other sequences.

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Methods

Nucleotide Sequence Data

Nucleotide sequences and information on host, sampling date, and location were obtained for all H6 nucleotide sequences from the National Center for Biotechnology Information Influenza Sequence Database and BioHealthBase (*24*,*25*) by using the following keywords: influenza A, H6, and avian host. All H6 sequences above the length of 1,650 nt were retained, and duplicate sequences of the same isolates were removed, leading to a total of 291 H6 sequences. These sequences were aligned with AlignX (Vector NTI version X; Invitrogen, Carlsbad, CA, USA), and a maximum likelihood phylogenetic tree was built using the *R* package ape (*26*). The tree was based on a substitution model that was first proposed by Felsenstein (*27*) and is now standard for maximum-likelihood estimation in most phylogenetic software packages (e.g., see p. 104 in *26*). This substitution model enables different rates for transitions and transversions, unequal base frequencies, and different substitution rates for wobble positions (*26*). The oldest H6 isolate was used as the root. The support of each bipartition was determined from 100-bootstrap samples. Other substitution models and neighbor-joining methods to construct phylogenetic trees produced very similar trees (H. zu Dohna, unpub. data).

Cross-Hemisphere Movement

Within the phylogenetic tree for the H6 gene, the 2 largest clades were determined and labeled as either North American or Eurasian, depending on where most isolates in each clade were found. All exceptions to this pattern, i.e., all isolates that clustered in a clade from a hemisphere that differed from their sampling location, were analyzed further. Following the analysis of Krauss et al. (*6*), these isolates were considered to have invaded a new hemisphere and will be referred to as invading isolates. The hemisphere to which the invading isolates belong genetically, as determined by the clade in which they cluster, will be referred to as the old hemisphere and the hemisphere from which they were isolated as the new hemisphere. All invading isolates were grouped into monophyletic invading subclades to estimate the times of invasion events.

For each invading subclade, 2 time points that bracket the most parsimonious invasion time were estimated. An invasion event that gave rise to a subclade must have happened after the last branching that led to descendents in the old hemisphere and before the time of the most recent common ancestor (TMRCA) for all descendents in the new hemisphere (Figure 1). These 2 time points were approximated for each subclade by estimating the TMRCAs for 2 sets of sequences, either including or excluding the most closely related sequence from the old hemisphere (Figure 1). This method assumes that invading isolates that are more closely related to each other than they are to other isolates from the old hemisphere descended from the same invasion event. This assumption is most parsimonious since there should be a low probability that the descendents of an invading virus reach a high enough density to be detected in bird samples several years later.

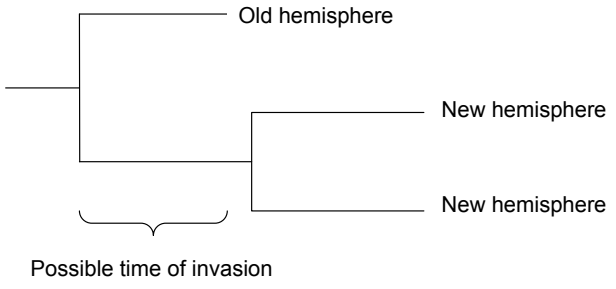

Figure 1. Possible period of invasion from the old hemisphere to the new hemisphere shown in a schematic tree with 2 isolates from the new and 1 from the old hemisphere.

TMRCAs were obtained by fitting a coalescent process to the gene sequence data, by using the Bayesian Monte Carlo Markov Chain package BEAST 1.4 (*28*). A general time-reversible substitution model with variable population size (*29*) was fitted to nucleotide data. To maximize conformity with model assumptions, only the wobble positions of conserved amino acids with 4-fold degenerate codons were chosen. The program returns sampled posterior distributions of each TMRCA estimate. The Markov chain was run for 5 million steps and parameters were sampled every 1,000th step.

The posterior distribution $f(t)$ of the time of invasion t was determined from all sampled pairs of the time of the earlier node and later node, t_{ei} and t_{li}, respectively:

$$f(t) = \frac{1}{c}\sum_i I(t_{ei} \le t \le t_{li})$$

where the sum was taken over all pairs i, I denotes an indicator variable that equals 1 if $t_{ei} \le t \le t_{li}$ and 0 otherwise, and c is a normalizing constant ensuring that $f(t)$ sums to 1 when summed over all times t. Additional analysis showed that the results were very similar when all nucleotide positions were included or when the HKY 85 substitution model (*30*) was chosen. However, the method described above produced the tightest posterior distributions for TMRCAs.

To determine whether invasion events had an effect on clade composition, the temporal change of the probability that an isolate was sampled from the North American clade was fitted by logistic regression using the statistical software *R* (*31*). The data were grouped by hemisphere

and host type (wild versus domestic birds) and analyzed for each group separately since wild and domestic birds were sampled differently. The wild bird analysis was based on individual isolates and the domestic bird analysis on the number of introductions into the domestic poultry species. Because the analysis relied on publicly available data, only introductions into domestic poultry that led to a sequence submission were analyzed. H6 sequences from domestic bird isolates in different years were counted as separate introductions. For all neuraminidase (NA) sequences that were part of a virus isolate whose H6 gene was classified as invader, the clade membership of the NA gene was determined as well. In addition, the proportion of H6 among all hemagglutinin sequences (any length, but only 1 sequence per isolate) from long-term wild bird survey sites in Alberta, Canada, and Ohio and Delaware, USA (*25*), was calculated per decade. These sites were chosen because AIV isolates were sampled from these sites following a standardized sampling regimen for more than a decade (*32*).

Results

The phylogenetic tree of all 291 full-length H6 gene sequences shows a clear division between the Eurasian and North American clades (online Technical Appendix Figure 1, available from www.cdc.gov/EID/content/15/7/1040-Techapp.pdf). Within the Eurasian clade there were 7 monophyletic subclades composed of strains isolated from North America (Figure 2). Six of these 7 subclades had bootstrap support above 90%. Five of these 7 subclades were closely related to isolates in Eurasia. Of these 5 subclades, 3 were most closely related to isolates in East Asia, and 2 were most closely related to clades in Northern Europe. All North American isolates of the 2 subclades most closely related to clades in Europe were found on the East Coast or in the Midwest region of the United States. Most of the isolates from the subclades closely related to isolates in Asia were found in Alberta, Canada, and the West Coast of the United States. The exceptions were 1 isolate from New Jersey and 1 from Delaware, which clustered in subclades with close relatives in Asia (Figure 2).

The estimated entry times of these 7 subclades overlapped in part, with the earliest times starting in the late 1970s and the latest around 2005 (Figure 3). The proportion of North American clade members among isolates from North American wild birds decreased from 100% in the 1980s to 20% in the 2000s (Figure 4; $p<0.0001$, logistic regression, residual deviance = 75, df = 106). Similarly, all H6 introductions into poultry species in North America with sequences submitted to the public databases were caused by viruses from the North American clade before 1998 and by viruses from the Eurasian clade after 1999 (Figure 4). All 38 H6 sequences isolated in North America after 2002

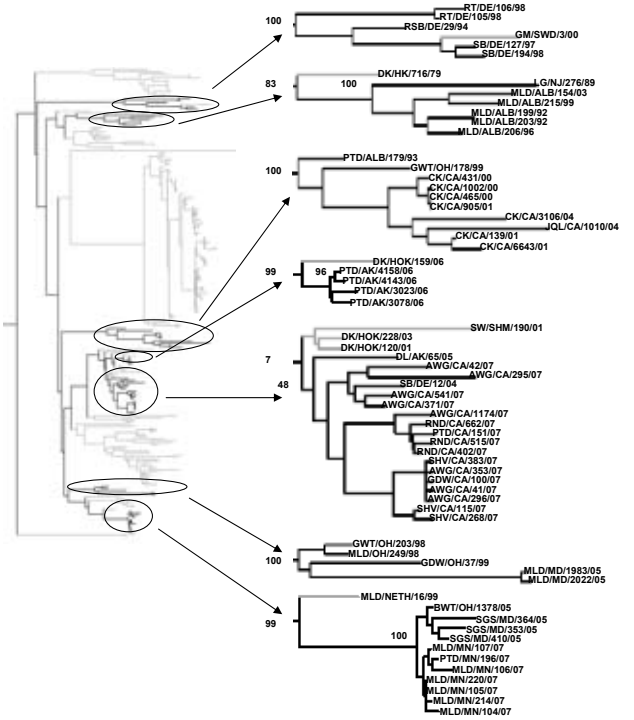

Figure 2. Eurasian clade of the phylogenetic tree for all full-length H6 sequences of avian influenza virus (excluding multiple sequences of the same isolate). Black branches indicate isolates from North America, and gray branches indicate isolates from Eurasia. The 7 subclades that invaded North America and their closest Eurasian related clade and bootstrap values are shown on the right. Abbreviations for strain names are listed in online Technical Appendix Table 1, available from www.cdc.gov/EID/content/15/7/1040-Techapp.pdf.

belong to the Eurasian clade. The change in clade composition among North American viruses is visible across the entire North American continent (online Technical Appendix Figures 2, 3). One subclade invaded Australia from North America but did not result in a significant impact on the clade composition over time in Eurasia (Figure 4, panel B). The increase of Eurasian H6 among North American H6 isolates did not coincide with an overall increase of H6 prevalence among AIV isolates reported in wild birds in North America (results not shown). All NA genes that were combined with invading H6 genes belonged to North American clades (online Technical Appendix Figures 4–7).

Discussion

A comprehensive phylogenetic analysis of all currently available full-length H6 gene sequences showed a major change in clade composition among isolates from North America. Seven subclades within the Eurasian clade were composed of isolates from North America. Isolates from these subclades started to appear in North America in the 1990s and became highly dominant among North

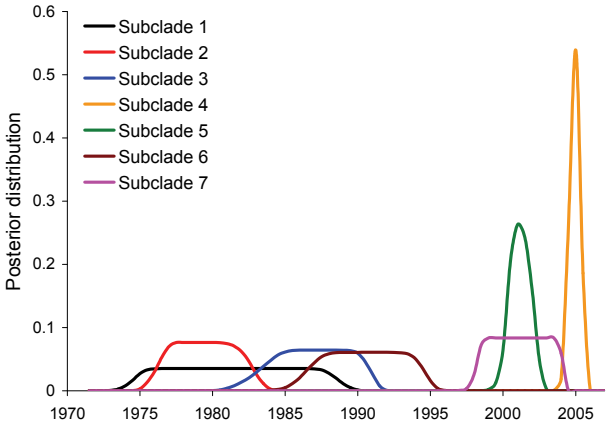

Figure 3. Posterior distribution of estimated invasion times of the 7 subclades of Eurasian avian influenza virus subtype H6 that invaded North America. Numbering of subclades corresponds to the order within the phylogeny of Figure 2 (top = 1, bottom = 7).

American isolates by the early 2000s. This pattern provides strong evidence for multiple invasions of North America by Eurasian H6 strains that led to the replacement of the North American H6 clade. Previous studies that addressed cross-hemisphere movement detected evidence for infrequent cross-hemisphere movement (*6*,*7*), but did not show the change of clade composition over time.

The closest native Eurasian relatives of the invading subclades indicated movement of H6 to North America from Northern Europe and East Asia. In addition, some evidence indicates possible movement back from North America to Eurasia (e.g., subclades 6 and 7 as shown in Figure 2 could have resulted from 1 movement from Europe into North America and a second movement from North America back to Europe). Although the phylogenetic analysis cannot assess the actual number of invasion events, it nevertheless indicates that several invasion events occurred. A main limitation of the data is that the sampling system was not consistent across time, space, and host species. For example, no H6 samples from the West Coast of North America are available before 2000; therefore, some of the decline in the proportion of the North American clade among H6 viruses could be attributed to an increase of samples from the West Coast, which contained only members of the Eurasian clade. However, even when isolates are grouped by region, the decline of the North American clade can still be observed (online Technical Appendix Figure 2).

Overall, the patterns suggest frequent movement between the hemispheres in the 1990s and 2000s. The deep division between the Eurasian and North American clades would not be expected if this frequent cross-hemisphere movement had occurred in the distant past. We therefore propose that cross-hemisphere virus movement increased in the last few decades. A formal test of this hypothesis would require estimating migration rates over time from phylogenetic data (*33*–*35*), while taking the limitations of the data into account, which is beyond the scope of the present paper. Comparing estimated time-varying virus migration rates with data on bird trade and wild bird migration might shed light on the mechanisms of cross-hemisphere movement.

That the sequences of the invading viruses did not cluster in a single monophyletic group suggests that the invasion events were not triggered by a single genetic change in the H6 gene. The pattern observed in the H6 gene could have been caused by the H6 gene spreading along with the expansion of another gene segment that reassorts rarely with the H6 gene. The analysis of the NA genes shows that no concurrent movement of NA genes occurred. However, we have not analyzed the internal genes. For a more complete understanding of AIV movement, the analysis developed here must be applied to all subtypes and gene segments.

Whether similar invasion events occurred for other gene segments or subtypes is unknown. Another study found that among AIVs, viruses of the H6 subtype have wider host ranges than other AIV subtypes (*13*). This could

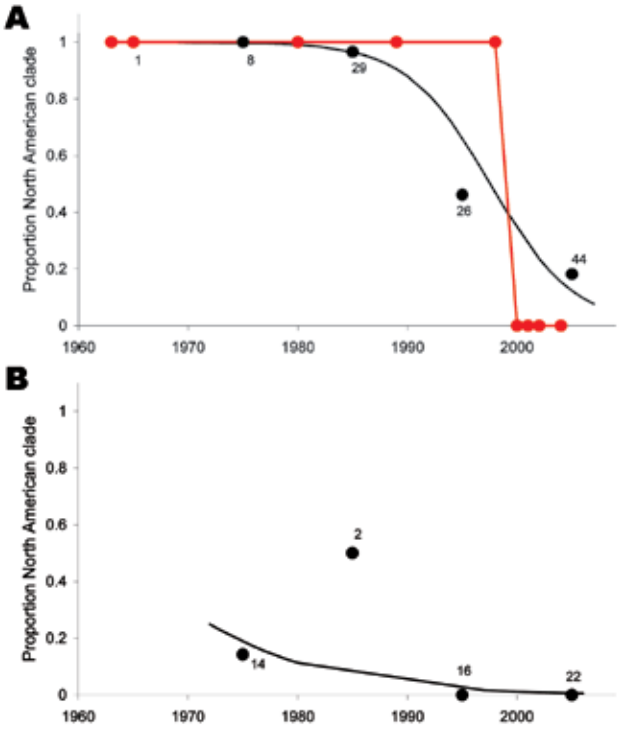

Figure 4. Proportion of viruses from the North American clade of avian influenza virus among wild bird isolates (blue) or poultry outbreaks (pink) in North America (A) or Eurasia (B). All poultry outbreaks in Eurasia were caused by viruses from the Eurasian clade (results not shown). Wild bird isolates are grouped by decade. Black circles show proportions of all isolates per decade (number of isolates per decade shown next to circles), black lines show fitted logistic regressions, and red circles show individual poultry outbreaks.

make H6 virus more prone to spread between and within hemispheres than other AIV subtypes.

Our study has shown that cross-hemisphere AIV movement can lead to a dramatic change in strain composition within a decade that affects an entire continent. We do not know whether expansion of the Eurasian H6 led to a change in risk for the poultry industry since H6 poultry isolates may not be well represented in public gene sequence databases. For example, H6 outbreaks have been documented in poultry operations in Minnesota in 12 different years from 1978 to 2005 (*36*), yet gene sequences of only 1 virus isolate from poultry outbreaks in Minnesota are available in GenBank. Although it is currently not possible to assess the risk posed to poultry by invading H6 AIV, the change in risk for the domestic poultry flocks clearly would be substantial if a similar invasion did occur for the highly pathogenic AIVs that are currently restricted to Eurasia.

This study corroborates a previous study (*6*) that found no evidence of cross-hemisphere invasions by entire virus genomes but detected only invasions of single gene segments that reassorted with other segments found in the new hemisphere. Thus, invasions can lead to new combinations of host range determining factors on different gene segments that were geographically separated before an invasion event. In general, invasions of the scope and speed as the instance documented here have the potential to strongly affect the risk posed by AIV to poultry and humans.

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Multiple Origins of Foot-and-Mouth Disease Virus Serotype Asia 1 Outbreaks, 2003–2007

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We investigated the molecular epidemiology of foot-and-mouth disease virus (FMDV) serotype Asia 1, which caused outbreaks of disease in Asia during 2003–2007. Since 2004, the region affected by outbreaks of this serotype has increased from disease-endemic countries in southern Asia (Afghanistan, India, Iran, Nepal, Pakistan) northward to encompass Kyrgyzstan, Tajikistan, Uzbekistan, several regions of the People's Republic of China, Mongolia, Eastern Russia, and North Korea. Phylogenetic analysis of complete virus capsid protein 1 (VP1) gene sequences demonstrated that the FMDV isolates responsible for these outbreaks belonged to 6 groups within the Asia 1 serotype. Some contemporary strains were genetically closely related to isolates collected historically from the region as far back as 25 years ago. Our analyses also indicated that some viruses have spread large distances between countries in Asia within a short time.

Foot-and-mouth disease virus (FMDV) is an *Aphthovirus* within the family *Picornaviridae* that infects domestic and free-ranging cloven-hoofed mammals. The virus occurs as 7 serotypes, and immunity after vaccination or after infection is type specific (*1–3*). Diversity is also apparent within serotypes, and phylogenetic studies have proved useful for tracing the origin of foot-and-mouth disease (FMD) outbreaks (*4*).

FMDV is highly contagious, and this, together with its ability to infect different hosts and to exist as multiple types and variants, makes FMD difficult to control and a severe constraint to international trade of livestock and their products. FMD is endemic to regions of South America and large areas of Africa and Asia, and it can readily cross international boundaries to cause epidemics in previously disease-free areas (*5*). High densities of ruminants and swine in Asia create potential reservoirs of virus maintenance and evolution not influenced by control measures. Intense trading of animals and their products from these reservoirs results in widespread dissemination of viruses within and outside this continent. Therefore, epidemiologic surveillance of FMD in Asia is essential for the timely detection of the emergence of new strains that could threaten neighboring countries (*6*) and for selecting the most appropriate vaccine strains for use and storage in emergency vaccine reserves (*7*).

Globally, FMDV serotypes O and the A are the most prevalent. However, Asia has its own unique serotype,

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Asia 1, first detected in samples collected in India in 1951 through 1952 (*8*) and Pakistan in 1954 (*9*). The primary serotype-endemic region for Asia 1 seems to be the Indian subcontinent (Afghanistan, India, Pakistan, Bhutan, Nepal), where outbreaks occur regularly, and some have speculated that this distribution is related to that of the Asian water buffalo (*Bubalus bubalis*). The serotype has been more sporadically reported from countries to the west or east; it has spread periodically into the Middle East and occasionally to Europe (*10–13*), but it has not been reported from Africa or the Americas. However, even in its endemic heartland, the Asia 1 serotype has normally been the cause of only a small proportion of cases compared with the proportion caused by serotypes O and A. For example, a study that reviewed FMDV in the West Bengal region of India described recovery of Asia 1 from only 15% of FMD cases examined between 1985 and 2002 (*14*). Similarly, in Southeast Asia, where serotypes O and A are prevalent every year, outbreaks due to Asia 1 have been reported only sporadically in the past 10 years; a recent gap in reporting occurred between 2002 and 2005 (Table; online Technical Appendix Table 1, available from www.cdc.gov/EID/content/15/7/1046-Techapp.pdf).

During 2004, evidence showed possible northward spread of the Asia 1 serotype; outbreaks were reported in Kyrgyzstan and Tajikistan. In early 2005, an outbreak was recorded in Hong Kong Special Administrative Region, People's Republic of China, which suggested that the virus might have crossed China. Later in 2005 and 2006, outbreaks of FMD Asia 1 were reported in several provinces and autonomous regions of China and in Mongolia and Eastern Russia (*15*). In 2005 and 2006, this serotype reappeared in Southeast Asia (Vietnam and Myanmar). This apparent upsurge in cases across a wide geographic area (Figure 1; online Technical Appendix Table 1) prompted the current collaborative study to determine the relationships between viruses, with the goal of better understanding the origin of these Asia 1 disease outbreaks.

Materials and Methods

Viruses

Clinical samples containing FMDV Asia 1 were received from Afghanistan, China, Hong Kong, Iran, Kyrgyzstan, Mongolia, Myanmar, Pakistan, Russia, and Tajikistan by the Food and Agriculture Organisation World Reference Laboratory for FMD (WRLFMD), FGI All-Russian Research Institute for Animal Health (Russian Federation), Lanzhou Veterinary Research Institute (China), Project Directorate on FMD (India), Plum Island Animal Disease Center (USA), and Pakchong Regional Reference Laboratory for FMD (Thailand) (online Technical Appendix Table 2).

RNA Extraction, Reverse Transcription–PCR, and DNA Sequencing

RNA extraction, 1-step reverse transcription–PCR (RT-PCR), and DNA sequencing were performed as previously described (*6*), except that the primer annealing temperature in the RT-PCR was 55°C. The primers used for RT-PCR and DNA sequencing are listed in online Technical Appendix Table 3. Specific methods used by each laboratory can be obtained on request.

Phylogenetic Analysis

Sequences of these viruses were compared with complete VP1 sequences of Asia 1 viruses stored in the WRLFMD database (n = 300) that have previously been published

Table. Countries that have reported outbreaks of foot-and-mouth disease virus serotype Asia 1, 2000–2008*

Country	2000	2001	2002	2003	2004	2005	2006	2007	2008
India	x	x	x	x	x	x	x	x	x
Pakistan		x	x	x	x	x			
Iran	x	x	x	x	x	x			
Nepal	x	x	x	x	x		x	x	
Bhutan			x						
Tajikistan					x				
Kyrgyzstan					x				
Afghanistan		x			x				
Turkey	x	x	x						
Myanmar	x	x				x			
Laos		x							
Thailand		x							
Vietnam						x	x	x	
People's Republic of China						x	x	x	x
Hong Kong						x			
Mongolia						x			
North Korea								x	
Russian Federation						x	x		

*Since 2005, countries are required to report a change in their foot-and-mouth epidemiologic situation only to the World Organisation for Animal Health.

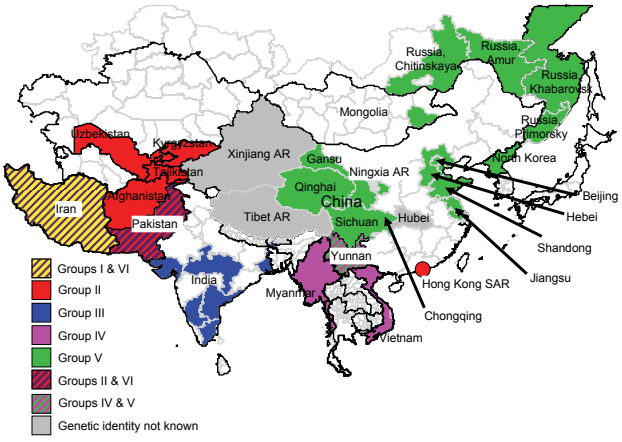

Figure 1. Origin (country and/or region) of isolates of foot-and-mouth disease virus serotype Asia 1 that were responsible for outbreaks in Asia during 2003–2007. The 6 different groups and their localities are indicated by different colors. AR, Autonomous Region; SAR, Special Administrative Region.

(*10*,*16*–*18*) or published in this article. Complete VP1 sequences were used to construct a midpoint-rooted neighbor-joining tree using the Kimura 2-parameter nucleotide substitution model as implemented in the program MEGA 4.0 (*19*). The robustness of the tree topology was assessed with 1,000 bootstrap replicates as implemented within the program. The topography of this tree was also checked by the maximum-parsimony (MEGA 4.0) and maximum-likelihood (TREE-PUZZLE 5.2) (*20*) methods, including a selection of isolates from each group to check the robustness of the topography. Subsequently, the sequences were ordered, based on their position in the neighbor-joining phylogenetic tree, and a matrix of percentage nucleotide differences was constructed by using MEGA 4.0. The matrix was imported into Excel 2007 (Microsoft Corporation, Redmond, WA, USA), and conditional formatting was used to identify relationships between sequences in the ranges 95%–100% and 90%–94.9%. The former value was used to group the most closely related virus sequences.

Results

The phylogenetic analysis of the complete VP1 gene sequences from isolates of serotype Asia 1 characterized in this study showed that recent viruses (isolated during 2003–2007) belonged to 6 different groups (I–VI) (Figure 2; online Technical Appendix Figure 1). These groups were defined by members of a group having 95%–100% nucleotide identity (online Technical Appendix Figure 2). All groups were supported by bootstrap values of 80%–100% (online Technical Appendix Figure 1) and were found by using alternative phylogenetic algorithms (maximum parsimony and maximum likelihood) (data not shown). Most virus groups were monophyletic. However, 1 group (VI) fell into 3 distinct lineages (a, b, c) and appeared to be ancestral to group II viruses (Figure 3, panel B). This grouping was also evident from the percentage identity matrix, in which the values between viruses in group VI and those in group II were 91.8%–95.9% (online Technical Appendix Figure 2). Relationships between groups II, III, and VI and between group IV and some unnumbered groups were also evident (online Technical Appendix Figure 2).

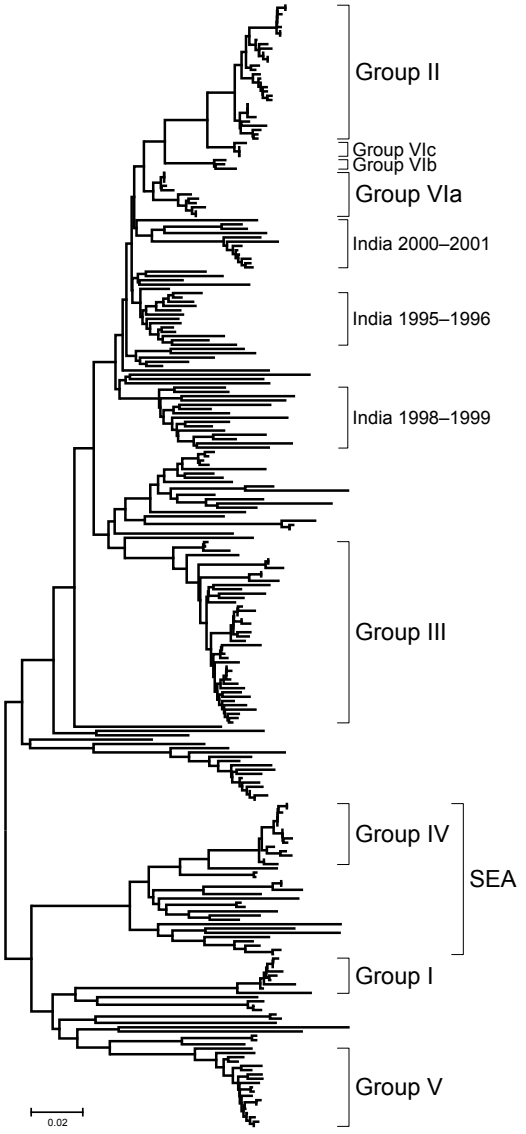

Figure 2. Midpoint-rooted neighbor-joining tree showing the relationships between the complete VP1 sequences of Asia 1 foot-and-mouth disease virus isolates studied. Only the tree structure is shown; details of the labeled groups are given in Figure 3. Scale bar indicates nucleotide substitutions per site. The complete tree with all viruses labeled is shown in online Technical Appendix Figure 1 (available from www.cdc.gov/EID/content/15/7/1046-Techapp.pdf). SEA, group of viruses found in only in Southeast Asia and Hong Kong.

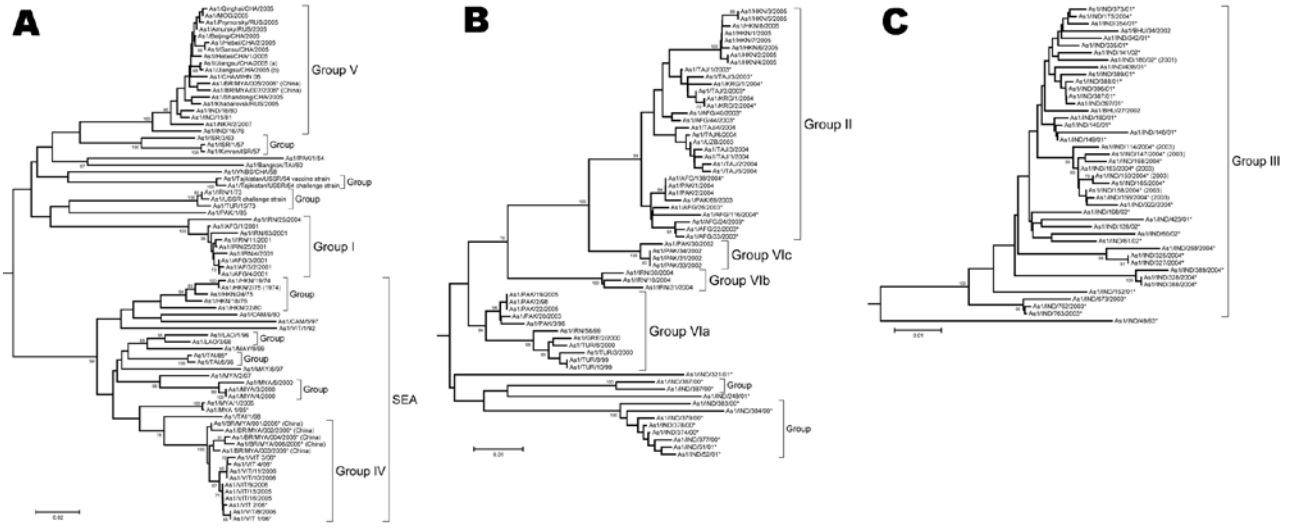

Figure 3. Midpoint-rooted neighbor-joining tree showing relationships between the foot-and-mouth disease Asia 1 viruses studied. A) groups I, IV, and V; B) groups II and VI; C) group III. Other groups of older (pre-2003) viruses sharing ≥90% nucleotide identity are indicated by the word "group" without any number. Only bootstrap values ≥70% are shown. Scale bars indicate nucleotide substitutions per site. SEA, group of viruses found in only in Southeast Asia and Hong Kong. *Indicates that the reference number is not one designated by the World Reference Laboratory for Foot-and-Mouth Disease.

Viruses that were circulating in Iran in 2004 belonged to 2 different groups (I and VI) (Figures 3, panels A and B). One isolate in group I, collected in Iran in 2004 (IRN/25/2004), was closely related to 8 viruses collected in Afghanistan and Iran in 2001. Other isolates collected in Iran during 2004 belonged to group VIb (e.g., IRN/30/2004) and had <7% nucleotide differences with isolates in group II that were collected in Uzbekistan (2003), Tajikistan (2003–2004), Afghanistan (2004), Kyrgyzstan (2004), Hong Kong (2005), and Pakistan (2002–2004). The report of FMDV Asia 1 in Hong Kong in 2005 was the first since 1980. Notably, the viruses collected in Uzbekistan, Tajikistan, Kyrgyzstan, and Hong Kong in 2003–2005 had <3% nucleotide differences, which suggests that the outbreaks were closely connected and that this virus may have spread a long distance in a short period; however, how this occurred remains unexplained.

Similarly, other viruses collected from Pakistan in 1998, 2003, and 2005 (group VIa) were closely related to viruses responsible for outbreaks in Iran (IRN/58/99), Turkey (TUR/3/2000 and TUR/6/2000), Armenia, Greece (GRE/2/2000), and Georgia from 1999 through 2001 (Figure 3, panel B) and from partial VP1 sequences (data not shown) (*10*,*12*). These data suggest that this epidemic may have originated in Pakistan. Previously, Asia 1 epidemics occurred in 1973 and 1983–1985. In 1973, the virus spread through Iran and Turkey without any traceable origin (*10*) (Figure 3, panel A), and in 1983–1985, genetically closely related viruses were found in many Middle Eastern countries, including Armenia, Azerbaijan, Bahrain, Georgia, Greece, Israel, and Lebanon (represented in online Technical Appendix Figure 1 by LEB/83 and GRE/1/84). However, the ultimate source of this virus strain was also not established (*4*,*10*). Surprisingly, FMD isolates collected in Pakistan in 2003 and 2005 (group VIa) were closely related to PAK/2/98, which had been isolated 5–7 years earlier, with 0.3% and 0.0% nucleotide differences, respectively (Figure 3, panel B). These differences would be consistent with a laboratory escape, use of an improperly inactivated vaccine, or laboratory contamination.

Group III contained only viruses that were collected in India during 2001–2004 and Bhutan (n = 2) during 2002 (Figure 3, panel C). Many other older virus lineages were evident in the phylogenetic analysis (online Technical Appendix Figure 1), showing the diversity of Asia 1 viruses in India. However, most of these lineages have not been detected outside the region, which suggests that endemic Asia 1 viruses rarely spread outside the Indian subcontinent. The reason is not understood.

Within group IV (Figure 3, panel A), FMD Asia 1 viruses responsible for outbreaks in China (Yunnan Province) and Vietnam in 2005 and 2006 were related to viruses originating from Southeast Asia that were collected in Thailand in 1998 and Myanmar in 2005. Viruses in group IV belonged to a larger, more diverse, group of viruses that were found in only in Southeast Asia and Hong Kong from 1974 through 2006 (indicated in Figures 2 and 3, panel A, as SEA). Only 2 viruses originating from Southeast Asia fell outside this supergroup, Bangkok/Thailand/60 (an old vaccine virus strain) and MYA/2/2001 (online Technical

Appendix Figure 1). The latter virus clustered with Indian virus isolates, suggesting a possible introduction into Myanmar from the west. In addition, in Myamar, several viruses belonging to 2 sublineages of group IV were detected in a relatively short period (1997–2000 and 2005; Figure 3, panel A), which implies that either multiple lineages are present or that multiple introductions have been made into that country.

FMDV isolates collected in different places in China, the Russian Federation, and Mongolia, during 2005–2006 (group V) were different from viruses isolated in Hong Kong in 2005 (group II) with 16.1%–17.2% nucleotide difference. Another virus belonging to group V has recently (2007) been identified as causing an outbreak of FMD in North Korea (NKR/2/2007) (Figure 3, panel A). The disease likely was introduced by importation of live calves from Liaoning Province, China. Of the 461 susceptible cattle, 431 (≈93%) were infected. All 461 susceptible cattle were destroyed. No cases were exhibited in swine, but 2,630 susceptible swine were destroyed (*21*). Viruses collected in the different provinces or regions of China, Russia, Mongolia, and North Korea during 2005–2007 were closely related to older viruses from India (Tamil Nadu) collected in 1976 and 1980–1981. The nucleotide differences between the Indian viruses and those from China, Mongolia, Russia, and North Korea (Figure 3, panel A) were 0.8%–4.6%, yet the viruses differed markedly from those that were collected more recently in India (group III; Figure 3, panel C) during 2003–2004 (n = 20); nucleotide difference was 12.8%–14.7%. No explanation is readily available, and further investigations need to be performed to determine the origin of the virus responsible for the outbreaks in China. Recently, 7 complete VP1 sequences of Asia 1 FMDV, originating from samples taken from cattle in 2006 in Yunnan Province close to the Myanmar border, were deposited in the public databases (accession nos. EU091342–EU091348; W. Zhang, Y. Hu, F. Zhang, unpub. data). An additional VP1 sequence from a virus from pigs in Sichuan Province in 2006 was also deposited (accession no. EU887277; H. Wang, X. Yang, H. Luo, unpub. data). Five of these sequences belonged to group IV and 2 belonged to group V (Figure 3, panel A), indicating movements of viruses between China and Southeast Asia and the presence of group V viruses in a more southerly distribution than has previously been reported.

Discussion

This phylogenetic study demonstrates that the viruses from groups II and V that have been responsible for FMD outbreaks in China appear to have spread large distances in a short time, although the means is unknown. The possibility of spread of viruses of these 2 groups beyond the border where they have been detected must be considered as a potential risk. Furthermore, the close relationships between some recent and older isolates within group V (India 1976–1981 vs. China/Mongolia/Russia/North Korea 2005–2007) and group VIa (Pakistan 1998 vs. Pakistan 2003–2005) raises the question of their origins, either as a result of an unusually slow evolutionary rate or as reintroductions of laboratory/vaccine virus strains.

In Asia, vaccination against FMD varies from country to country; generally, only cattle and water buffalo are vaccinated. Various vaccine strains are used in the region, and vaccines are produced either by large pharmaceutical companies or by national or regional FMD vaccine laboratories. Vaccine matching studies are performed in various FMD reference laboratories on an ad hoc basis, and reference reagents for all the vaccine strains are not always available. This situation requires improvement.

These studies suggest rapid spread of FMD viruses across Asia, but the means by which the viruses are moved has rarely been determined. The spread of some of these FMDV Asia 1 lineages across large parts of Asia, and occasionally outside Asia, demonstrates the continuing need for active surveillance to be improved in Asia to provide real-time monitoring of virus evolution and to disclose more effectively the links between outbreaks. The means of virus transport needs also to be defined, taking into consideration the role played by large antelope populations in central Asia. This information is needed as a prerequisite for further development of regional control programs. India, Pakistan, and China, with their large livestock populations, are expected to play a major role in FMD control in this part of the world.

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Clusters of Multidrug-Resistant *Mycobacterium tuberculosis* Cases, Europe

Isabelle Devaux, Kristin Kremer, Herre Heersma, and Dick Van Soolingen

Molecular surveillance of multidrug-resistant tuberculosis (MDR TB) was implemented in Europe as case reporting in 2005. For all new MDR TB cases detected from January 2003 through June 2007, countries reported case-based epidemiologic data and DNA fingerprint patterns of MDR TB strains when available. International clusters were detected and analyzed. From 2003 through mid-2007 in Europe, 2,494 cases of MDR TB were reported from 24 European countries. Epidemiologic and molecular data were linked for 593 (39%) cases, and 672 insertion sequence *6110* DNA fingerprint patterns were reported from 19 countries. Of these patterns, 288 (43%) belonged to 18 European clusters; 7 clusters (242/288 cases, 84%) were characterized by strains of the Beijing genotype family, including the largest cluster (175/288 cases, 61%). Both clustering and the Beijing genotype were associated with strains originating in eastern European countries. Molecular cluster detection contributes to identification of transmission profile, risk factors, and control measures.

Prevalence of multidrug-resistant tuberculosis (MDR TB), i.e., TB resistant to at least rifampin and isoniazid, is increasing, particularly in some areas of Europe (*1*). From 1999 through 2002, the median prevalence of MDR TB in new case-patients was at critical levels (>6.5%) in specific regions of the world, including the Baltic states and other eastern European countries (*2*). In 2004, the Russian Federation, China, and India accounted for 62% of the estimated global MDR TB cases (*3*). The increases in prevalence and incidence of MDR TB are most likely caused by inadequate treatment regimens (*4*). Recently, public health awareness about MDR TB has been reenforced by the occurrence of extensively drug-resistant (XDR) TB outbreaks associated with HIV, particularly in South Africa (*5,6*). Surveillance of drug resistance, based on annual case reporting of drug susceptibility tests, has been ongoing in Europe since 1998 and includes annual reporting of MDR TB cases after the start of treatment (*7*). In the 1990s, molecular methods became available and have allowed researchers to study issues in the epidemiology of TB (*8*). Strains of the *Mycobacterium tuberculosis* Beijing genotype, which constitute a group of genetically closely related strains (*9*), have been associated with high levels of drug resistance in countries of the former Soviet Union (FSU), including Latvia (*10*) and Estonia (*11*); these strains have been of low prevalence (6%–7%) in Western Europe (*12*).

Public health officials are concerned about possible emergence and transmission of MDR TB strains across Europe (cross-border migration), but no system is in place to identify whether MDR TB strains are shared (clustered) among European countries. Risk factors for possible clustering have yet to be determined. When the drug resistance surveillance project began, data on MDR TB cases were reported in aggregated format at the European level and could not be linked to molecular data identified from individual MDR TB cases. Also, a need existed to further identify the association between Beijing genotype strains or *M. tuberculosis* strains of other genotypes and MDR TB in Europe.

Molecular surveillance of MDR TB was developed in Europe in recent years, first, in a pilot phase as part of the European Concerted Action on Tuberculosis project and, prospectively, since 2005, with the MDR TB project presented in this paper (*13*). The objectives of this project were to identify molecular clusters of MDR TB cases reported in

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>1 European country, to describe epidemiologic risk factors associated with MDR TB cases, and to initiate cluster investigations at the national level to prevent cross-border transmission.

In this article, based on surveillance data, we describe the main epidemiologic and demographic characteristics of MDR TB cases reported from January 2003 through June 2007 in 24 European countries. We also describe characteristics of the main European clusters and identify risk factors for clustering and association with the Beijing strain of *M. tuberculosis*.

Materials and Methods

Data Collection and Management

In 2005, all 53 countries of the World Health Organization's European region were invited to participate in the MDR TB surveillance project by Euro TB and the National Institute for Public Health and the Environment (RIVM). Countries were encouraged to provide both epidemiologic and genotyping data on MDR TB cases reported since January 1, 2003. However, countries that could provide only epidemiologic data were also included in the surveillance project. National surveillance institutions sent quarterly updates of individual and anonymous data on MDR TB cases reported since 2003 to EuroTB, according to a standardized data file specification (www.eurotb.org/mdr_tb_surveillance/pdf/DFS_NSI_short.pdf). Each patient had a unique record identifier by country. Common definitions of variables were used by participating countries, including demographic, clinical, and genotyping information. In 18 countries, the country of origin was defined as the country of birth. Because of confidentiality constraints, country of birth was not reported for some countries. Country of citizenship was used to qualify the origin of patients for 2 countries. Either country of birth or country of citizenship was used in 3 countries, and information on geographic origin was unavailable in 1 country. A patient had to be reported again (with a new identifier) when a new MDR *M. tuberculosis* isolate was obtained ≥24 months after the previously reported MDR TB isolate. However, the possibility of a case being reported twice in the same country or in 2 countries was low.

National laboratories sent insertion sequence (IS) *6110* restriction fragment length polymorphism (RFLP) patterns from available MDR TB strains collected since 2003 to the RIVM every 3 months, either as a Bionumerics bundle (Applied Maths, Sint-Maartens-Latem, Belgium) or as a scanned image (*14*). To be included in the project, laboratories had to perform drug susceptibility testing and participate in an international quality assurance program. For quality assurance, either participating laboratories represented supranational reference laboratories of the World Health Organization and the International Union against Tuberculosis and Lung Disease and participated in the yearly proficiency testing for isoniazid, rifampin, streptomycin, and ethambutol (*15,16*), or laboratories had their external quality assurance conducted by such a reference laboratory. For 1 country, drug susceptibility testing was performed in a laboratory abroad. More details (e.g., on drug susceptibility test methods) can be found in the 2006 EuroTB report (*17*).

European cluster information was communicated by the RIVM (Bilthoven, the Netherlands) to EuroTB on a quarterly basis. The final database was maintained by EuroTB and included 3 sections: A) demographic and clinical variables, B) cluster information on the basis of molecular patterns at the country level, and C) European cluster information. Data from sections A and B were matched according to a patient code; data from sections B and C were matched using the strain code attributed at the national level.

Analysis of Genotyping Data

IS*6110* RFLP was the recommended genotyping method (*18*) to report IS*6110* RFLP patterns to RIVM. For most countries, genotyping was performed locally; for 2 countries, genotyping was performed at RIVM. A European cluster was defined as ≥2 MDR TB cases with *M. tuberculosis* isolates that shared identical IS*6110* RFLP patterns in ≥2 countries (*18*). A national cluster included cases diagnosed in a single country. Determination of a national cluster (a cluster including MDR TB cases diagnosed in 1 participating country) could be based on 3 typing methods: IS*6110* RFLP typing (*18*), spoligotyping (*19*), and/or typing that used mycobacterial interspersed repetitive units with variable numbers of tandem repeats (*20*).

In each laboratory, quality control of the molecular typing practices and computer-assisted analysis was included as described in the standardized methods (*14*). The DNA fingerprint patterns of MDR TB strains received at the RIVM were submitted to the molecular database managed by the RIVM (MDRTBase) by using the Bionumerics software (Applied Maths), and the database manager performed a quality check. The IS*6110* RFLP patterns newly added to the MDRTBase were then compared with all other patterns stored in this database. The MDR TB strains were classified as clustered (included in a European cluster) or "unique" (not included in a European cluster). In addition to specifying European cluster reports, the database manager also specified strains belonging to the Beijing genotype family of *M. tuberculosis* by compariing them with the 19 reference RFLP patterns of Beijing strains described by Kremer et al. (*21*).

Statistical Data Analysis

Factors associated with clustering or infection with a Beijing genotype strain were determined by the unadjusted and adjusted logistic regression model using SAS software (SAS Institute, Cary, NC, USA). The explanatory variables were demographic (sex, age, origin) and clinical (pulmonary vs. extrapulmonary TB, TB history) characteristics of MDR TB cases. XDR TB was studied as an explanatory variable for patients with drug susceptibility results meeting the definition of XDR TB (*22*) in both cluster and strain analyses. The proportion of clustering among Beijing strains was compared with the proportion of clustering among strains of other genotypes.

Because of the limited numbers per category, "unknown" categories were removed from all variables included in the model, as was the age category "<15 years" and the category "other" for the variable country of origin. Analyses, including clustering and the XDR variable, were performed in the univariate model only because of the variables' strong association with origin in the Baltic states (χ^2 97.1, $p<0.001$) and other countries of the FSU (χ^2 20.5, $p<0.001$).

Results

Country Participation, Number of MDR TB Cases Reported, and Clustering of MDR TB Cases

Twenty-four countries from the European Union and western Europe plus Croatia and Macedonia participated in the MDR TB project (Table 1). Epidemiologic data on 2,494 MDR TB cases reported from January 2003 through July 2007 were sent to EuroTB. The Baltic states (Estonia, Latvia, and Lithuania) reported 1,616 cases, representing 65% of total cases. In some countries, epidemiologic data were reported for <3 years (Germany, Lithuania, Poland, Romania, and Spain).

Laboratories from 19 countries sent 672 IS*6110* RFLP patterns to the RIVM, 593 of which also had epidemiologic information and were linked to the epidemiologic database maintained by EuroTB (Table 1; Figure 1). Linkage of molecular and epidemiologic data was not possible for data from the United Kingdom and for data on a few cases from 6 other countries. The average proportion of MDR TB cases documented with both epidemiologic and genotyping data in 18 countries was 593/1,523 (39%), varying from 9% to 100%. After we removed data from 2 countries with low data completeness for genotypes (Germany and

Table 1. Number of diagnosed MDR TB cases reported in 24 European countries and number and proportion of genotyped MDR TB strains, as reported to the MDR TB surveillance project, by year, January 2003 through June 2007*

	No. cases reported					No. genotyped strains reported	
Country	2003	2004	2005	2006 and 2007	Total	All	With data (% total cases)
Belgium† (EU)	9	14	7	1	31	15	15 (48)
Croatia	2	1	2	–	5	5	5 (100)
Cyprus (EU)	2	0	1	–	3	–	–
Czech Republic (EU)	15	13	4	6	38	11	5 (13)
Denmark (EU)	0	0	5	–	5	4	4 (80)
Estonia (EU)	96	80	72	–	248	228	228 (92)
Finland (EU)	3	0	2	2	7	7	7 (100)
France (EU)	53	53	46	–	152	63	63 (41)
Germany (EU)	–	–	101	–	101	17	16 (16)
Ireland (EU)	1	2	3	2	8	3	3 (38)
Israel	18	15	12	–	45	39	39 (87)
Italy (EU)	45	18	17	3	83	25	25 (30)
Latvia (EU)	188	208	160	156	712	–	–
Lithuania (EU)	–	318	338	–	656	56	56 (9)
Macedonia, FYR	4	1	6	4	15	–	–
Netherlands (EU)	17	10	7	–	34	44	34 (100)
Norway (EU)	2	4	3	2	11	7	3 (27)
Poland (EU)	–	–	1	16	17	–	–
Romania (EU)	–	25	25	–	50	–	–
Slovenia (EU)	1	0	1	1	3	2	2 (67)
Spain (EU)	–	28	22	–	50	50	50 (100)
Sweden (EU)	7	7	4	3	21	21	18 (86)
Switzerland	9	9	3	4	25	25	20 (80)
United Kingdom (EU)	68	52	54	–	174	50	–
Total	540	858	896	200	2,494	672	593 (39)

*MDR TB, multidrug-resistant tuberculosis; EU, European Union; –, not available; FYR, Former Yugoslav Republic of Macedonia.
†Includes only cases detected at the start of treatment.

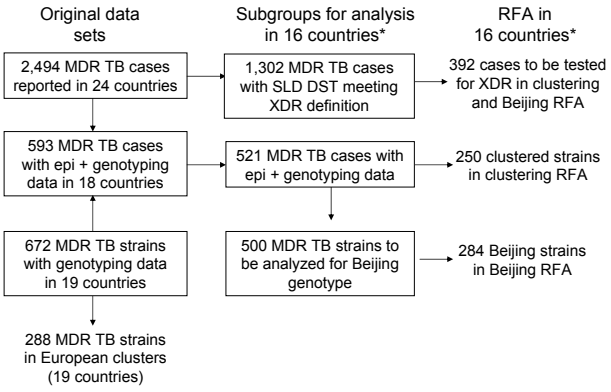

Figure 1. Description of MDR TB cases in Europe selected for data analysis, January 2003–July 2007. RFA does not include data from Germany and Lithuania. MDR TB, multidrug-resistant tuberculosis; SLD, second-line drug; DST, drug-susceptibility test; RFA, risk factor analysis. *Countries: Belgium, Czech Republic, Denmark, Estonia, Finland, France, Germany, Ireland, Israel, Lithuania, the Netherlands, Norway, Spain, Sweden, Switzerland, and the United Kingdom.

Lithuania), we found that the average proportion of MDR TB cases with both epidemiologic and genotyping data was 68%. Individual data on second-line drug tests for 2 to 5 second-line drugs included in the XDR TB definition (*22*) were reported for 1,302 cases by 16 countries from January 2003 through July 2007 (Figure 1).

Of the 672 MDR TB strains with IS*6110* RFLP patterns reported, 288 (43%) were identified as belonging to European clusters (Table 2; Figure 1). The proportion of MDR TB strains included in European clusters was <20% in 7 countries (Croatia, France, Italy, Slovenia, Spain, Switzerland, and the United Kingdom). A large proportion (38%) of the RFLP patterns submitted to the molecular database originated from Estonia, where 80% (183/228) of the strains clustered. The number of MDR TB strains identified in national clusters was 170 among 330 strains (51%) reported in 13 of the 19 countries with genotyping data. In these 13 countries, the proportion of intercountry clustering was 28%.

Description of MDR TB Clusters

Eighteen distinct European clusters (range 2–175 cases) were identified in 16 countries (Table 3). Clusters E0051, E0054, E0055, E0057, E0063, E0066, and E0069 were characterized by Beijing genotype strains, comprising 242/288 (84%) clustered MDR TB cases. The characteristics of the 4 largest clusters are described below.

Cluster E0051

The largest cluster, E0051, consisted of 175 MDR TB cases reported in 12 countries. Of these 175 cases, 148 (85%) were reported in Estonia. No significant differences were found between case-patients included in cluster E0051 and those included in other clusters for sex, age category, TB history, and XDR TB (χ^2 test, $p>0.05$). A large proportion (119/148, 81%) of the case-patients reported in Estonia also originated from this country (Figure 2). The proportion of case-patients who had never had TB (either no diagnosis or no treatment) was high (90/148, 61%). Thirty-three cases (22%) were identified as XDR TB. Eight of the 175 case-patients in cluster E0051 were reported in Israel, and all 8 originated from FSU countries (6 from the Russian Federation, 1 from Georgia, and 1 from Kazakhstan); all were pulmonary patients and alive at the time of diagnosis. No information was available on previous TB diagnosis or treatment. None of the 8 MDR TB cases reported in Israel were XDR. The 6 case-patients reported in Lithuania also originated from that country. Five of these case-patients were known to have had a previous diagnosis of TB; XDR TB was not diagnosed in these case-patients. The 3 case-patients reported in Belgium originated from the Russian Federation (n = 2) and from Georgia. Their TB history was unknown. For the 2 case-patients reported in Finland, origin was unknown. One of the 2 case-patients reported in Switzerland was from Armenia; the origin of the other case-patient was unknown. The 2 case-patients reported in the Netherlands and Sweden originated from the Russian Federation.

Table 2. Number of genotyped MDR TB strains in 19 European countries, with cluster status, by country, January 2003 through June 2007*

Country	Genotyped strains	European clusters, no. (%)	National clusters, no. (%)
Belgium†	15	7 (47)	7 (47)
Croatia	5	0	5 (100)
Czech Republic	11	3 (27)	–
Denmark	4	1 (25)	1 (25)
Estonia	228	183 (80)	–
Finland	7	3 (43)	5 (71)
France	63	10 (16)	24 (38)
Germany	17	8 (47)	9 (53)
Ireland	3	1 (33)	–
Israel	39	18 (46)	32 (82)
Italy	25	0	–
Lithuania	56	18 (32)	43 (77)
Netherlands	44	11 (25)	17 (39)
Norway	7	2 (29)	2 (29)
Slovenia	2	0	2 (100)
Spain	50	7 (14)	14 (28)
Sweden	21	9 (43)	9 (43)
Switzerland	25	3 (12)	–
United Kingdom	50	4 (8)	–
Total	672	288 (43)	170 (51)

*MDR TB, multidrug-resistant tuberculosis; –, not available.
†Includes only cases detected at the start of treatment.

Table 3. Number of multidrug-resistant tuberculosis strains in 16 European countries, by cluster and by country, January 2003 through June 2007

Country	Cluster no. (E00--)																		Total
	51*	54*	55*	53†	64	68	60	66*	70†	57*	63*	67†	56	59	61	62	65	69*	
Estonia	148	18	13					2			2								183
Israel	8	3	2		2			2					1						18
Lithuania	6	3	1		4		2										1	1	18
Netherlands	1	4	1	2		1									1	1			11
France				6			1		1	1				1					10
United Kingdom	1		1	1								1							4
Sweden	1	2	1						1	1		1		1		1			9
Germany	1	3	2							1	1								8
Belgium	3			2									1		1				7
Spain						5			1									1	7
Switzerland	2			1															3
Czech Republic							2										1		3
Finland	2											1							3
Norway	1		1																2
Denmark									1										1
Ireland	1																		1
Total	175	33	22	12	6	6	5	4	4	3	3	3	2	2	2	2	2	2	288

*Caused by Beijing genotype strains.
†Caused by low-copy strains; the insertion sequence *6110* restriction fragment length polymorphism pattern of cluster E0053 consisted of 4 bands; cluster E0070, 3 bands; and cluster E0067, 1 band.

Cluster E0054

Twenty-eight of the 33 case-patients included in the second largest cluster, E0054, had a known origin. All but 1 (from Israel) originated from FSU countries (17 originating and reported in Estonia).

Cluster E0055

Eighteen of the 22 case-patients included in cluster E0055 had a known origin; 13 were reported in Estonia, 2 in Israel, 1 in Lithuania, 1 in the Netherlands, and 1 in Sweden. All originated from FSU countries, except the case-patient in the Netherlands, who originated from Sri Lanka.

Cluster E0053

Cluster E0053 (non-Beijing) included 10 cases with known origin. Five case-patients originated from the country of report, and 5 others originated from Africa (2 from Côte d'Ivoire, 2 from Nigeria, and 1 from Mali). Because this cluster was characterized by an *M. tuberculosis* strain containing 4 copies of IS*6110*, it could possibly be subdivided if additional DNA typing methods are used. As indicated in Table 3, two other clusters were also caused by low–copy-number strains: clusters E0070 (4 cases) and E0067 (3 cases).

Risk Factors for Clustering Among MDR TB Isolates

Risk factors for clustering were analyzed for 521 cases documented with both molecular and epidemiologic data reported by 16 countries. Lithuania and Germany were excluded from this analysis due to incomplete genotyping data. Among these 521 cases, 250 (48%) belonged to European clusters and 271 (52%) had isolates with unique DNA fingerprint patterns (Figure 1). The results of univariate and multiple logistic regression analyses to determine factors associated with clustering are presented in Table 4.

Univariate analysis showed that origin from the Baltic states or other FSU countries, age category 45–64 years, and pulmonary tuberculosis were significantly associated with clustering. Among these variables, only origin

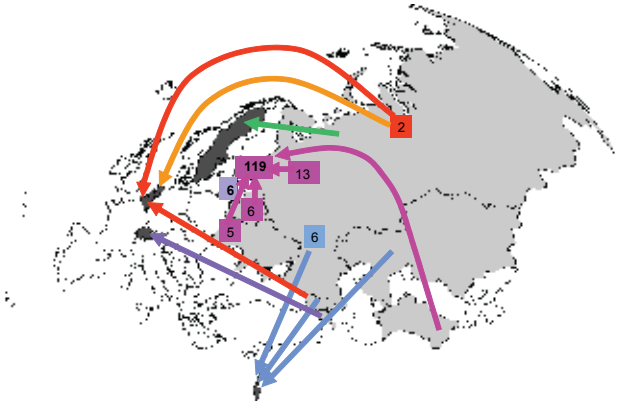

Figure 2. Origin and country of report for the largest European cluster of multidrug-resistant tuberculosis cases (cluster E0051). The cases were reported in 12 European countries (see Table 3). Five countries did not have information about the origin of the cases reported there. For cases reported in the countries shaded in dark gray, the base of the arrow shows where case-patients originated (1 case per arrow, unless otherwise indicated). The 6 case-patients reported in Lithuania were also born there. Of the 148 case-patients reported in Estonia, 119 were also born there. The remaining case-patients reported in Estonia were originally from Russia (13), Belarus (6), Ukraine (5), Lithuania (1) and Turkmenistan (1); origin was unknown for 3 case-patients.

Table 4. Predictors for clustered MDR TB strains compared with unique strains among 521 cases reported in 16 European countries, January 2003 through June 2007*

Variable	MDR TB strains		Unadjusted OR (95% CI)	Adjusted† OR (95% CI)
	No. patients	% Clustered		
Sex				
Male	347	48	1	1
Female	170	49	1.0 (0.7–1.5)	1.1 (0.6–1.9)
Age				
15–44	324	43	1	1
45–64	143	61	2.1 (1.4–3.1)	1.3 (0.7–2.3)
≥65	36	53	1.5 (0.7–3.0)	2.8 (0.8–9.4)
Origin				
Baltic states	188	81	**21.9 (10.6–44.9)**	**25.1 (9.9–64.0)**
Other FSU countries‡	99	61	**7.7 (3.7–16.1)**	**8.7 (3.2–23.2)**
Other European countries	72	17	1	1
Africa	71	24	1.6 (0.7–3.6)	1.1 (0.4–3.5)
Site of disease				
Pulmonary	473	50	**2.4 (1.2–4.9)**	0.5 (0.2–1.6)
Extrapulmonary	41	29	1	1
Previous TB				
Yes	163	54	1.0 (0.6–1.4)	1.1 (0.6–1.9)
No	248	55	1	1
XDR TB				
Yes	51	82	**4.1 (1.9–8.7)**	–
No	341	53	1	–

*TB, tuberculosis; MDR, multidrug-resistant; OR, odds ratio; CI, confidence interval; FSU, former Soviet Union; XDR, extensively drug-resistant. **Boldface** indicates significance.
†For all other factors in the model except XDR TB.
‡Former Soviet Union countries: Azerbaijan, Belarus, Georgia, Kazakhstan, Kyrgyzstan, Moldova, Russia, Tajikistan, Ukraine, and Uzbekistan.

from the Baltic states (adjusted odds ratio [OR] 25.1, 95% confidence interval [CI] 9.9–64.0) or other FSU countries (adjusted OR 8.7, 95% CI 3.2–23.2) remained strongly associated with clustering in the multivariate model. The proportion of XDR TB cases was significantly higher among clustered strains than among unique strains (OR 4.1, 95% CI 1.9–8.7).

Risk Factors for TB Characterized by Strains of the Beijing Genotype among MDR TB Cases

Analysis to identify Beijing genotype strains was possible for 500 of the 521 MDR TB strains with both epidemiologic and genotyping data (data from Germany and Lithuania were excluded). Of these 500 strains, 284 (57%) were identified as the Beijing genotype and 216 (43%) were non-Beijing genotypes (Figure 1). Univariate analysis demonstrated that origin from the Baltic states or other FSU countries, age categories 45–64 years and ≥65 years, and pulmonary TB were significantly associated with strains of the Beijing genotype (Table 5). Among these variables, origin from Baltic states and other FSU countries remained strongly associated with Beijing genotype strains in the multivariate analysis. Proportions of clustering and XDR TB were significantly higher among Beijing strains than among other genotype strains, (OR 19.6, 95% CI 12.3–31.2, and OR 5.5, 95% CI 2.1–14.3, respectively). After exclusion of the largest cluster, E0051 (n = 166 cases with epidemiologic data), characterized by a Beijing strain, the association between clustering and the Beijing genotype was 5× less but still quite strong for 334 MDR TB cases with genotyping data (OR 4.5, 95% CI 2.7–7.6).

Discussion

Molecular surveillance of MDR TB cases in Europe showed several large molecular clusters (also including XDR TB cases). The largest cluster (175 cases) was mainly localized in Estonia (information to be completed for Lithuania and Latvia). Origin from the Baltic states and other FSU countries was strongly associated with clustering. The proportion of XDR TB was high among clustered strains. The high proportion of MDR TB case-patients without reported TB history suggests circulation of primary MDR strains in these countries. Three of the 18 European clusters were caused by low-copy strains and may represent false clustering. However, because these clusters consisted of only a few cases, the proportion of European clustering found in this project (43%) is probably only slightly (≤3%) overestimated.

A high proportion (55%) of genotyped MDR TB strains belonged to the Beijing genotype family. Strains of the Beijing genotype family were mainly reported for case-patients originating from the Baltic states and other FSU countries, where the prevalence of this genotype is also high in the general population (*12*). The 3 largest clusters

Table 5. Predictors for TB caused by Beijing strains among 506 cases reported in 16 European countries, January 2003 through June 2007*

Variable	MDR TB strains		Unadjusted OR (95% CI)	Adjusted† OR (95% CI)
	No. patients	% Beïjing strains		
Sex				
Male	333	58	1	1
Female	164	55	0.9 (0.6–1.3)	1.1 (0.5–2.4)
Age				
15–44	313	53	1	1
45–64	142	67	**1.7 (1.1–2.6)**	1.0 (0.5–2.1)
≥65	30	70	2.5 (1.1–5.5)	2.3 (0.5–10.4)
Origin				
Baltic states	188	92	**154.5 (54.0–442.0)**	**173.8 (45.5–663.6)**
Other FSU countries‡	99	75	**39.7 (14.4–109.5)**	**32.2 (8.6–120.8)**
Other European countries	71	6	1	1
Africa	71	10	1.5 (0.4–4.9)	1.1 (0.2–5.5)
Site of disease				
Pulmonary	455	59	**3.4 (1.7–6.6)**	0.7 (0.1–3.3)
Extrapulmonary	40	30	1	1
Previous TB				
Yes	156	64	1.1 (0.8–1.7)	1.0 (0.5–2.0)
No	245	62	1	1
Clustering				
Yes	250	87%	**19.0 (12.0–30.3)**	–
No	250	26%	1	
XDR TB				
Yes	51	90	**5.5 (2.1–14.3)**	–
No	341	62	1	

*TB, tuberculosis; MDR, multidrug-resistant; OR, odds ratio; CI, confidence interval; FSU, former Soviet Union; XDR, extensively drug-resistant. **Boldface** indicates significance.
†For all other factors in the model except XDR TB.
‡Former Soviet Union countries: Azerbaijan, Belarus, Georgia, Kazakhstan, Kyrgyzstan, Moldova, Russia, Tajikistan, Ukraine, and Uzbekistan.

were characterized by strains of the Beijing genotype family (84% of the clustered MDR TB cases).

Two hypotheses may explain the occurrence of the large clusters in Europe that were detected in this project. The first hypothesis is direct person-to-person transmission. This hypothesis can be verified by cluster investigation of the cases (by using contact-tracing data if available) in countries with cases reported in clusters. Verifying that the isolates, especially of the largest clusters, represent a single strain is important and can be done by contact investigations and application of an additional typing method such as the newly standardized 24-loci variable number tandem repeat (VNTR) typing (*23*). The second hypothesis is that genetically highly related strains are responsible for most MDR TB cases in Europe and that only a part of the cases in these clusters is associated with direct person-to-person transmission. In both scenarios, conditions could be improved by reinforcing case management (including healthcare access), following up on treatment (especially drug compliance), and increasing social support. Adequate infection control measures should also be ensured in healthcare facilities. The second scenario underlines that more fundamental research (including detailed research on DNA repair, fitness, and transmissibility) is needed to better understand changes in the bacterial population structure of TB, including the drug-susceptible bacterial population and the ongoing evolutionary development of *M. tuberculosis*.

This study was limited by a lack of completeness of the genotyping information and, to a lesser extent, of epidemiologic information. Data completeness varied by country. For some countries (Norway, the United Kingdom, the Netherlands, and Germany), the molecular data reported represent only a fraction of the molecular data available because these countries also used alternative DNA typing methods, which were not included in this project. Other countries have not yet implemented genotyping. Low data completeness in some countries could have affected the proportion of clustered versus unique strains, possibly introducing a bias in the interpretation of the risk factor analysis. However, the proportion of MDR TB cases submitted with both epidemiologic and genotyping information from 16 (68%) countries is promising, considering that IS*6110* RFLP typing is technically demanding. This proportion could probably be improved in the future with the implementation of the recently standardized VNTR typing (*23*).

Another limitation of this study was that the identification of MDR TB clusters was based on genotype cluster-

ing. Therefore, person-to-person transmission could not be proven. Ideally, transmission dynamics should be confirmed by conventional contact investigations to link patients to contact persons (*24*). For the main clusters, EuroTB correspondents (from the countries where these clusters have been identified) have received the identification numbers of the clustered MDR TB cases and were able to communicate with colleagues from other countries concerned by the same cluster and exchange information on case histories. However, for the largest MDR TB cluster (E0051), a special field investigation of cases would be necessary and would require international support and coordination. With the limitations of this study, the findings represented here may not be representative of all MDR TB cases in Europe.

MDR TB cases included in clusters should be investigated (including contact-tracing data) in countries where they have been identified to increase knowledge about cross-border transmission of MDR TB and XDR TB and to identify routes of transmission and additional risk factors for these transmission pathways. The outputs of the investigations will enable interventions that prevent further transmission of MDR TB and XDR TB caused by increasing migration between European countries. Standards should be developed to conduct these investigations, including mechanisms to inform concerned national authorities when a molecular cluster is detected and a method for linking epidemiologic and genotyping data at the European level. Delays between diagnosis of MDR TB cases and cluster detection should be reduced to facilitate appropriate actions in the concerned countries and to avoid international transmission of these strains. International collaboration in cluster investigations should be encouraged

Results of this project are preliminary because data are not complete for all countries. Since March 2008, European surveillance of MDR TB has been undertaken by the European Centre for Disease Prevention and Control. A molecular component has been added in this surveillance program. Ideally, this project should be extended to other European Union countries and neighboring countries that could provide genotyping data.

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Relapse Associated with Active Disease Caused by Beijing Strain of *Mycobacterium tuberculosis*[1]

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The role of microbial factors in outcomes of tuberculosis treatment has not been well studied. We performed a case–control study to evaluate the association between a Beijing strain and tuberculosis treatment outcomes. Isolates from patients with culture-positive treatment failure (n = 8) or relapse (n = 54) were compared with isolates from randomly selected controls (n = 296) by using spoligotyping. Patients with Beijing strains had a higher risk for relapse (odds ratio [OR] 2.0, 95% confidence interval [CI] 1.0–4.0, p = 0.04) but not for treatment failure. Adjustment for factors previously associated with relapse had little effect on the association between Beijing strains and relapse. Beijing strains were strongly associated with relapse among Asian–Pacific Islanders (OR 11, 95% CI 1.1–108, p = 0.04). Active disease caused by a Beijing strain was associated with increased risk for relapse, particularly among Asian–Pacific Islanders.

Approximately 2%–5% of patients with tuberculosis (TB) treated with contemporary short-course treatment either fail to respond to therapy or recurrent TB can develop in these patients after they complete therapy, despite assurance of adherence through supervised treatment (*1*,*2*). In settings with a high prevalence of disease, a substantial percentage of recurrent cases are caused by reinfection with another strain of *Mycobacterium tuberculosis* (*3*,*4*). However, reinfection is an uncommon cause of recurrent disease in settings in which the prevalence of active TB is low (*5*). Several studies have evaluated risk factors for recurrent TB, which are severity of the radiographic manifestations of disease (presence of cavitation [*1*,*6*–*8*], extent of pulmonary involvement [*1*,*6*,*8*], or the presence of silicosis [*9*]), microbial load at diagnosis (*7*), and 2-month sputum culture positivity as an indicator of the early response to therapy (*1*,*6*,*7*).

Whether aspects of the infecting strain of *M. tuberculosis* might affect treatment outcomes has not been well studied. Studies early in the time of chemotherapy found that *M. tuberculosis* strains from patients who responded well to isoniazid monotherapy seemed to be somewhat less virulent in a guinea pig model of active TB (*10*). However, this line of investigation was not pursued in the context of response to multidrug therapy. DNA fingerprinting techniques enable classification of *M. tuberculosis* isolates into genotype families. The Beijing genotype family has received considerable attention because of its association with drug resistance (*11*,*12*). Furthermore, the Beijing family may be rapidly spreading in some areas (*13*–*15*). Studies from Vietnam (*16*) and Singapore (*17*) showed that active disease caused by a Beijing strain was associated with an increased risk for recurrent TB after completion of treatment.

Recent studies have shown associations among *M. tuberculosis* strains, geographic regions, and human populations, which suggest that specific strains of *M. tuberculosis* coevolved with human subpopulations (*18*–*20*). However, with increased population mobility, there has been greater mixing of *M. tuberculosis* strains and human subpopulations. The interaction between these factors (the bacillary strain and the race/ethnic background of the patient), may

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affect response to TB treatment (*21*). We used isolates from a large multicenter clinical trial (Tuberculosis Trials Consortium [TBTC] Study 22) (*1*) to evaluate the association between active disease caused by a Beijing strain and TB treatment outcomes. We also explored whether this association was affected by the race/ethnicity of the patient.

Methods

Study Population

TBTC Study 22 was a randomized trial comparing once-a-week rifapentine plus isoniazid with twice-a-week rifampin plus isoniazid during the last 4 months of short-course treatment for drug-susceptible pulmonary TB (*1*,*22*). Adults were enrolled from sites in the United States and Canada from 1995 through 1998. Before enrollment, all patients had completed an initial 2 months of treatment with isoniazid, rifampin, pyrazinamide, and ethambutol (or streptomycin). All TB treatment was supervised (directly observed therapy).

Sputum cultures were obtained monthly during treatment. Failure was defined as a positive culture ≥4 months of treatment. Relapse was defined as a positive culture during the 2-year follow-up after completion of therapy. Paired isolates from the time of enrollment and the time of suspected treatment failure or relapse underwent insertion sequence (IS) *6110* fingerprinting (*1*,*23*), and an endpoint review committee determined whether the positive culture was caused by cross-contamination, relapse, or reinfection. TBTC Study 22 was reviewed and approved by the Institutional Review Boards of CDC and participating sites. The present analysis of isolates from that study was reviewed and found to be research that did not include human patients.

Selection of Case-Patients and Controls

We evaluated the association between active disease caused by a Beijing strain and TB treatment outcomes by using a nested case–control study of isolates from participants in TBTC Study 22. Because it is likely that the risk factors for treatment failure and relapse are different among persons with HIV co-infection, we limited this case–control analysis to HIV-negative participants (n = 1,004). Case-patients were participants who adhered to study therapy and had culture-positive treatment failure or relapse caused by the initial infecting strain of *M. tuberculosis*. Controls were selected through simple random sampling of participants who completed treatment and had 2 years of follow-up (3, 6, 9, 12, 18, and 24 months after treatment completion) with no clinical or microbiologic evidence of treatment failure or relapse. Cases and controls were not matched for any demographic, clinical, or radiographic characteristics.

Laboratory Methods

Isolates from cases and controls underwent spoligotyping (*24*) as modified by Cowan et al. (*25*). Beijing strains were defined as isolates with a spoligotype showing the absence of spacers 1–34 and presence of at least 3 spacers among spacers 35–43 (*26*,*27*). To evaluate possible associations between *M. tuberculosis* lineages, as defined by large sequence polymorphisms and treatment outcomes, isolates were assigned by spoligotype pattern to previously described lineages (*20*,*21*).

Statistical Analyses

The number of cases was determined from the parent clinical trial; 4 controls were randomly selected for each case. Race/ethnicity was determined by site staff who used categories defined in the United States TB surveillance system (*28*).

The definitions and primary objective of this case–control analysis were formulated before data analysis. The initial analysis evaluated the association between active disease caused by a Beijing strain and treatment failure or relapse. To enable comparisons with previous studies (*16*,*17*), we then analyzed relapse alone as the outcome. We subsequently analyzed the relationships between race/ethnicity, active disease caused by a Beijing strain, and treatment outcomes. Associations between active disease caused by a Beijing strain and baseline characteristics and treatment outcomes were evaluated by using χ^2 analysis. Associations with treatment outcomes were adjusted in multivariate logistic regression models for factors previously associated with failure and relapse. These factors were white race, being underweight, pulmonary cavitation, bilateral pulmonary involvement, and 2-month sputum culture positivity (*1*). Because having been randomly assigned rifapentine as treatment was associated with an increased risk for relapse in univariate analyses of TBTC Study 22 (*1*), we conducted a secondary analysis in which treatment assignment was forced into multivariate models of risk for poor treatment outcomes.

Results

Of the 1,004 HIV-negative participants in TBTC Study 22, there were 8 instances of treatment failure and 61 cases of relapse. Of these 69 cases of adverse TB treatment outcomes, isolates from 64 patients were successfully genotyped (Figure). Of the 930 study participants who did not experience treatment failure or relapse, 76 did not complete therapy and 172 did not complete follow-up. Of the remaining 687 patients, 296 (43%) were randomly selected as controls for this analysis; their 296 baseline isolates were successfully genotyped (Figure).

Demographic and clinical characteristics of cases and controls are provided in Table 1. As in the entire study

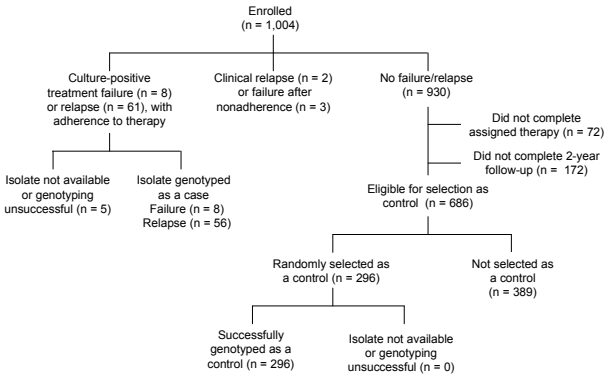

Figure. Selection of case-patients and controls, Tuberculosis Trials Consortium Study 22. The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants.

cohort, white race, being underweight, pulmonary cavitation, bilateral pulmonary involvement, and 2-month sputum smear and culture positivity were associated with an increased risk for treatment failure and relapse among the participants of this case–control analysis (Table 1). Patients with active disease caused by a Beijing strain had an increased risk for treatment failure or relapse (15 [23%] of 64 cases vs. 42 [14%] of 296 controls; odds ratio [OR] 1.9, 95% confidence interval [CI] 0.9–3.6, p = 0.07). When analyzed as separate endpoints (Table 2), treatment failure was not associated with active disease caused by a Beijing strain (OR 0.9, 95% CI 0.1–7.2, p = 1.00), but relapse was significantly associated (OR 2.0, 95% CI 1.0–4.0, p = 0.04). Active disease caused by the Indo-Oceanic lineage was associated with a lower risk for treatment failure or relapse, although the small number of isolates from that lineage (n = 25) and the lack of any cases of treatment failure or relapse among patients with active disease caused by this lineage precluded further evaluation of this association.

Adjustment for the 5 clinical, radiographic, and microbiologic risk factors for treatment failure and relapse in the parent study had little effect on the OR of active disease being caused by a Beijing strain and relapse (adjusted OR 2.2, 95% CI 1.0–5.0, p = 0.05) (Table 3). Similarly, treatment assignment (rifapentine vs. rifampin) had little effect in a multivariate model into which this factor was forced (adjusted OR for active disease caused by Beijing strain 2.3; 95% CI 1.0–5.3, p = 0.04).

We next evaluated the effect of race/ethnicity on the association between relapse and active disease caused by a Beijing strain (Table 4). Asian–Pacific Islanders were not at increased risk for relapse in the entire TBTC Study 22 cohort or in this nested case–control analysis (OR 0.6, 95% CI 0.2–1.4, p = 0.23). However, Asian–Pacific Islanders who had active disease caused by a Beijing strain were at increased risk for relapse (OR 11, 95% CI 1.0–108, p = 0.04) (Table 4).

We further evaluated the association between relapse and active disease caused by a Beijing strain among race/ethnicity groups by using stratified analysis. In an analysis limited to Asian–Pacific Islanders, adjustment for other risk factors for relapse had little effect on the association between relapse and active disease caused by a Beijing strain (adjusted OR 15.8, 95% CI 1.3–192, p = 0.03) (Table 5). Among other race/ethnicity groups, there was no association between relapse and active disease caused by a Beijing strain (Table 5).

Discussion

Using isolates from participants in a large prospective trial of supervised intermittent treatment, we found that active disease caused by a Beijing strain of *M. tuberculosis* was associated with a 2-fold increased risk for adverse TB treatment outcomes. The relationship between Beijing strains and treatment outcomes was driven by relapse because of their greater frequency and an apparent lack of any association between Beijing strains and treatment failure. Adjustment for the clinical, radiographic, and microbiologic risk factors for treatment failure and relapse and for treatment assignment in TBTC Study 22 had little effect on the association between active disease caused by a Beijing strain and the risk for relapse. In an exploratory analysis of the relationships of the effect of race/ethnicity on the association between Beijing strains and treatment outcomes, Asian–Pacific Islander patients were at increased risk for relapse if they had active disease caused by a Beijing strain, albeit with wide CIs around the risk estimate (adjusted OR 13.9, 95% CI 1.3–164).

Two other studies have evaluated the association between the Beijing genotype and relapse. A case–control analysis from Vietnam reported an adjusted OR of 3.2 for treatment failure or relapse among patients with active disease caused by a Beijing strain (*16*), and a cohort study from Singapore found an adjusted OR of 2.64 for relapse associated with Beijing strains (*17*). Our study provides additional support for the hypothesis that active disease caused by a Beijing strain is associated with increased risk for relapse (adjusted OR 2.2, 95% CI 1.0–4.9). The use of a nested case–control analysis of carefully characterized patients in a clinical trial enabled us to evaluate the relationship between Beijing strains and treatment outcomes in analyses adjusted for host factors previously associated with an increased risk for relapse. We were also able to eliminate effects of other possible confounding factors, such as treatment duration and adherence to treatment. Additionally, because reinfection could obscure the relationship between infection with a Beijing strain and the risk for relapse, we were able to remove cases of reinfection from

Table 1. Characteristics of case-patients and controls, Tuberculosis Trials Consortium Study 22*

Characteristic	Case-patients (treatment failure or relapse), n = 64†	Controls (patients cured), n = 296†	Odds ratio (95% confidence interval)	p value
Demographic				
Age, y, mean (SD)	42 (13)	44 (14)	1.0 (0.97–1.01)	0.51
Men	54 (84)	214 (72)	2.1 (1.0–4.3)	0.05
Treatment				
Rifapentine, 1×/wk	40 (63)	151 (51)	1.6 (0.9–2.8)	0.10
Rifampin, 2×/wk	24 (37)	145 (49)		
Ethnic origin				
Non-Hispanic white	22 (34)	43 (15)	3.1 (1.7–5.7)	0.0002
Non-Hispanic black	25 (39)	127 (43)	0.9 (0.5–1.5)	0.57
Hispanic	9 (14)	71 (24)	0.5 (0.2–1.1)	0.08
Asian–Pacific Islander	6 (9)	45 (15)	0.6 (0.2–1.4)	0.23
Native American	2 (3)	10 (3)	0.9 (0.2–4.3)	0.92
Birthplace				
United States or Canada	48 (75)	200 (68)	1.4 (0.8–2.7)	0.24
Mexico	5 (8)	36 (12)	0.6 (0.2–1.6)	0.32
Europe	2 (3)	4 (1)	2.4 (0.4–13.1)	0.31
Southeast Asia	2 (3)	6 (2)	1.6 (0.3–7.9)	0.59
Western Pacific	4 (6)	33 (11)	0.5 (0.2–1.6)	0.24
Other	3 (5)	17 (6)	0.8 (0.2–2.8)	0.74
Baseline clinical features				
Fever	50/62 (81)	166/289 (57)	3.1 (1.6–6.0)	0.0007
Sweats	42/63 (67)	162/287 (56)	1.5 (0.9–2.7)	0.14
Cough	61 (95)	256/294 (87)	3.0 (0.9–10.1)	0.06
Underweight‡	38 (59)	82 (28)	3.8 (2.2–6.7)	<0.0001
Sputum smear positive	55 (86)	193/292 (66)	3.1 (1.5–6.6)	0.002
Baseline chest radiographic features				
Cavitation	54 (84)	146/287 (51)	5.2 (2.6–10.6)	<0.0001
Bilateral pulmonary involvement	50 (78)	155/293 (53)	3.2 (1.7–6.0)	0.0002
Two-month sputum analysis				
Smear positive	16 (25)	30/285 (11)	2.8 (1.4–5.6)	0.002
Culture positive	33 (54)	48/267 (18)	5.4 (3.0–9.7)	<0.0001
Mycobacterium tuberculosis lineage/family				
East Asian/Beijing	15 (23)	42 (14)	1.9 (0.9–3.6)	0.07
Euro-American	47 (73)	221 (75)	0.9 (0.5–1.7)	0.84
Indo-Oceanic	0	25 (8.5)	0.08 (0.01–1.4)	0.01
East African	1 (1.6)	3 (1.0)	1.6 (0.16–15.1)	0.54
Unclassified lineage	1 (1.6)	5 (1.7)	0.9 (0.11–8.04)	1.00

*The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants.
†Except for age, values are no. (%) or no. positive/no. tested (%).
‡Less than 10% below ideal bodyweight at diagnosis.

this analysis. That adjustment for other risk factors for relapse, such as pulmonary cavitation and being underweight, had little effect on the association is additional evidence that active disease caused by a Beijing strain increases the risk for relapse.

After evolving in eastern Asia, the Beijing family of *M. tuberculosis* has spread around the world (*26*,*29*). Why might active disease caused by a Beijing strain confer an increased risk for relapse? Laboratory studies have suggested that Beijing strains may be better adapted for intracellular

Table 2. Association between treatment failure or relapse and active disease caused by Beijing vs. other genotypes of *Mycobacterium tuberculosis*, Tuberculosis Trials Consortium Study 22*

Outcome	Disease caused by Beijing genotype, n = 57	Disease caused by other genotype, n = 303	Odds ratio (95% CI)	p value
Cure (n = 296)	42 (14)	254 (86)	1.9 (0.9–3.6)	0.07
Failure or relapse (n = 64)	15 (23)	49 (77)		
Failure (n = 8)	1 (13)	7 (88)	0.9 (0.1–6.3)	1.00
Relapse (n = 56)	14 (25)	42 (75)	2.0 (1.0–4.0)	0.04

*The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants. CI, confidence interval.

Table 3. Association between active disease caused by a Beijing genotype of *Mycobacterium tuberculosis* and relapse, adjusted for clinical risk factors, Tuberculosis Trials Consortium Study 22*

Characteristic	Univariate analysis		Multivariate analysis	
	Odds ratio (95% CI)	p value	Odds ratio (95% CI)	p value
Infected with Beijing genotype	2.0 (1.0–4.0)	0.05	2.2 (1.0–4.9)	0.07
Non-Hispanic white race/ethnicity	3.3 (1.7–6.1)	<0.01	3.0 (1.4–6.7)	<0.01
Underweight at tuberculosis diagnosis	4.7 (2.6–8.6)	<0.01	3.7 (1.8–7.2)	<0.01
Pulmonary cavitation	5.0 (2.4–10.7)	<0.01	3.2 (1.4–7.5)	0.01
Bilateral pulmonary disease	2.9 (1.5–5.7)	<0.01	1.8 (0.9–4.0)	0.12
Two-month sputum culture positivity	4.7 (2.6–8.7)	<0.01	2.4 (1.2–4.9)	0.01

*The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants. CI, confidence interval.

growth (*30*,*31*) and are more virulent in animal models of TB, perhaps by evading immune responses (*32*,*33*). Furthermore, an altered DNA repair enzyme in Beijing isolates that confers a mutator phenotype may confer greater flexibility to respond to adverse conditions (*34*), such as those posed by multidrug therapy.

The possibility that Beijing strains evade immune responses and are more virulent is generally borne out in studies with humans. The Beijing family is associated with extrapulmonary TB (*35*,*36*). That Beijing strains have been implicated in many outbreaks of TB suggests that they may be more efficiently transmitted or have an enhanced ability to progress to active disease than do other strains. In a study in Russia, active disease caused by a Beijing strain was associated with more severe radiographic manifestations of pulmonary TB (*11*). However, 2 smaller studies did not find an association between Beijing strains and radiographic severity of pulmonary TB (*37*,*38*). Because of its case–control design, our study cannot directly address the unresolved question of whether active disease caused by a Beijing strain is associated with radiographic severity of disease (such a comparison would require a cohort study design). However, adjustment for cavitation and bilateral pulmonary involvement did not affect the association between Beijing strains and relapse in our study. This finding suggests that the mechanism of the association between Beijing strains and relapse is not mediated by radiographic severity of disease.

Our study suggests that the increased risk for relapse associated with active disease caused by a Beijing strain may be related to the race/ethnicity of the patient; risk for relapse was higher for persons of Asian–Pacific Islander descent. There was also a trend toward increased risk for relapse among black patients infected with a Beijing strain, according to univariate analysis (OR 2.9, 95% CI 1.0–8.5; p = 0.07), although this trend was not retained in adjusted analysis (Table 5). It is notable that the population in which the Beijing genotype conferred the greatest risk for relapse was of Asian–Pacific Islander race/ethnicity and that the Beijing genotype evolved in eastern Asia. Coevolution of the Beijing genotype among persons of East Asian descent may have selected factors that contribute to transmissibility and a decreased response to therapy.

Our study has at least 4 limitations. First, the cohort from which this nested case–control analysis was drawn was composed of patients who enrolled in a randomized trial, who differed from the broader patient population at study sites. One clear bias in the study population is that TBTC Study 22 was limited to patients with drug-susceptible TB. Therefore, we cannot evaluate the association between Beijing strains and drug resistance. Second, despite the size of the study cohort, our case–control analysis had limited statistical power to detect associations, particularly in exploratory analyses of the relationships between *M. tuberculosis* genotype, race/ethnicity of the host, and the risk for relapse. Third, race/ethnicity was defined by using broad categories developed for the United States census and used in the TB surveillance system. However, these categories are crude approximations of the genetic background of patients. For example, the category Asian–Pacific Islander includes several distinct ethnic groups that have substantial differences in genetic backgrounds. We did not have access

Table 4. Association between active disease caused by a Beijing genotype of *Mycobacterium tuberculosis* and relapse, by racial/ethnic background, Tuberculosis Trials Consortium Study 22*

Race/ethnicity	Patients with disease that relapsed		Relapse odds ratio (95% CI)	p value
	Infected with a Beijing strain, no. positive/no. tested (%)	Not infected with a Beijing strain, no. positive/no. tested (%)		
Non-Hispanic white (n = 63)	3/11 (27)	17/52 (33)	0.8 (0.2–3.3)	0.73
Non-Hispanic black (n = 148)	6/22 (27)	15/126 (12)	2.8 (0.9–8.2)	0.06
Hispanic (n = 79)	1/7 (14)	7/72 (9.7)	1.5 (0.2–15)	0.70
Asian–Pacific Islander (n = 50)	4/16 (25)	1/34 (2.9)	11 (1.1–108)	0.04

*The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants. Participants of Native American race/ethnicity (n = 12) were not included because none were infected with a Beijing strain; 2 Native American participants had disease that relapsed. CI, confidence interval.

Table 5. Multivariate analyses of association between active disease caused by a Beijing strain of *Mycobacterium tuberculosis* and relapse among race/ethnicity groups while controlling for other risk factors for relapse, Tuberculosis Trials Consortium Study 22*

	Asian–Pacific Islander (n = 50)		Non-Hispanic black (n = 148)		Non-Hispanic white (n = 63)		Hispanic (n = 79)	
Characteristic	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
Infected with Beijing strain	15.8 (1.3–192)	0.03	1.8 (0.5–6.5)	0.35	1.0 (0.1–7.7)	0.98	1.0 (0.1–13)	0.97
Underweight at tuberculosis diagnosis	3.1 (0.3–34)	0.35	2.9 (0.8–6.3)	0.15	11 (2.4–48)	<0.01	4.6 (0.9–24)	0.07
Pulmonary cavitation	2.1 (0.1–33)	0.60	4.0 (0.8–19)	0.09	2.7 (0.5–15)	0.25	6.6 (0.7–61)	0.09
Bilateral pulmonary disease	5.2 (0.4–69)	0.21	1.6 (0.5–4.8)	0.44	1.2 (0.2–9.9)	0.84	2.1 (0.3–15)	0.46
Two-month sputum culture positivity†	–	–	3.3 (1.1–9.7)	0.03	3.5 (0.6–20)	0.16	4.6 (0.5–40)	0.17

*The Tuberculosis Trials Consortium Study enrolled patients during 1995–1998. Participants in the case–control study were selected from among 1,004 HIV-infected participants. OR, odds ratio; CI, confidence interval.
†Not included for Asian–Pacific Islander patients because none were culture positive at 2 months.

to human genetic material that would enable precise delineation of the genetic backgrounds of the trial's participants. Fourth, we did not adjust for multiple comparisons, and our study should be viewed as an exploratory, hypothesis-generating analysis.

In summary, our study offers additional evidence that a common genotype of *M. tuberculosis*, the Beijing family, is associated with increased risk for relapse after completion of supervised short-course TB treatment. The finding that the population at greatest risk for relapse if they had active disease caused by a Beijing genotype was persons of Asian–Pacific Islander race/ethnicity suggests that the coevolution of this bacterial strain and the human population may have selected factors that confer a poor response to therapy.

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Dr Burman is medical director of the Infectious Diseases Clinic of Denver Public Health and an investigator in the Tuberculosis Trials Consortium. His primary research interest is in the design and implementation of randomized clinical trials to improve the treatment of TB and HIV disease.

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Frequency and Evolution of Azole Resistance in *Aspergillus fumigatus* Associated with Treatment Failure[1]

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Azoles are the mainstay of oral therapy for aspergillosis. Azole resistance in *Aspergillus* has been reported infrequently. The first resistant isolate in Manchester, UK, was detected in 1999. In a clinical collection of 519 *A. fumigatus* isolates, the frequency of itraconazole resistance was 5%, a significant increase since 2004 (p<0.001). Of the 34 itraconazole-resistant isolates we studied, 65% (22) were cross-resistant to voriconazole and 74% (25) were cross-resistant to posaconazole. Thirteen of 14 evaluable patients in our study had prior azole exposure; 8 infections failed therapy (progressed), and 5 failed to improve (remained stable). Eighteen amino acid alterations were found in the target enzyme, Cyp51A, 4 of which were novel. A population genetic analysis of microsatellites showed the existence of resistant mutants that evolved from originally susceptible strains, different *cyp51A* mutations in the same strain, and microalterations in microsatellite repeat number. Azole resistance in *A. fumigatus* is an emerging problem and may develop during azole therapy.

Invasive aspergillosis in immunosuppressed patients is difficult to diagnose, is problematic to treat, and results in a high mortality rate (*1*). Chronic and allergic pulmonary and sinus aspergillosis are increasingly recognized in numerous clinical settings. Treatment with itraconazole, voriconazole, and, recently, posaconazole is the backbone of therapy for these conditions because azoles are the only licensed class of oral drugs for treatment of aspergillosis (*2,3*). Amphotericin B and caspofungin are licensed intravenous agents for invasive aspergillosis but have limited utility for chronic and allergic aspergillosis.

Itraconazole resistance in *Aspergillus* spp. was first reported in 1997 in 3 clinical isolates obtained from California in the late 1980s (*4*); since then, only a few clinical cases have been published (*5–9*). The emergence of itraconazole resistance alone is of concern, but widespread azole cross-resistance would be devastating.

The primary mechanism of resistance described for *A. fumigatus* clinical isolates is mutation in the target protein. The *cyp51A* gene encodes the target of azoles, lanosterol 14α-demethylase, and this enzyme catalyzes a step in the biosynthetic pathway of ergosterol (an essential cell membrane component of filamentous fungi). Mutations in the open reading frame of the *cyp51A* gene can result in structural alterations to the enzyme, which in turn may inhibit binding of drugs. Mutational hotspots confirmed to cause resistance have been characterized in the gene at codons 54 (*6,10–13*), 220 (*6,14,15*), and 98 (*16–18*). Other mutations in the *cyp51A* gene have been reported, and additional resistance mechanisms have been postulated (*11,19,20*). The environmental or antifungal pressures driving azole resistance are unclear because few clinical azole-resistant *Aspergillus* strains have been studied in any detail; many reports simply describe individual patient cases. In this study, we

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investigated the frequency of *A. fumigatus* itraconazole resistance in a referral laboratory collection, defined the azole cross-resistance pattern, identified mutations in the *cyp51A* gene, and investigated any epidemiologic links between resistant isolates.

Materials and Methods

Isolates

Isolates deposited in the Regional Mycology Laboratory Manchester (RMLM) culture collection (between 1992 and 2007) were identified as *A. fumigatus* by macro- and micromorphologic characteristics. All isolates were screened for growth at 50°C, thus confirming *A. fumigatus* and excluding *A. lentulus*. Aspergilli were subcultured onto Sabouraud glucose agar (Oxoid, Basingstoke, UK) for 48 h at 37°C. Thirty-four azole-resistant and 5 susceptible isolates from 17 patients were studied from the RMLM collection (prefixed F); 36 isolates were respiratory specimens, 1 was cerebral, and 2 were from unknown sites. In addition, 18 azole-resistant isolates from a single aspergilloma case-patient (prefixed A, patient 3) collected at autopsy were also investigated.

Patients

Pertinent details from patients were extracted from the clinical records. All but 6 were under the care of 1 investigator (D.W.D.). Information was collected on underlying disease(s), type of aspergillosis, antifungal treatment, azole plasma levels, and characteristics of therapeutic failure.

Susceptibility Testing

Susceptibilities were determined by a modified European Committee on Antimicrobial Susceptibility Testing (EUCAST) method (*21*). The modification was a lower final inoculum concentration (0.5×10^5 as opposed to 1–2.5 $\times 10^5$ CFU/mL). Isolates were tested at a final drug concentration range of 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015 mg/L against itraconazole (Research Diagnostics Inc, Concord, MA, USA), voriconazole (Pfizer Ltd, Sandwich, UK), posaconazole (Schering-Plough, Kenilworth, NJ, USA), and amphotericin B (Sigma, Poole, UK). RPMI-1640 (Sigma) was supplemented to 2% glucose (Sigma). Inocula were prepared in phosphate-buffered saline with 0.05% Tween 80 (Sigma); *Aspergillus* spores were counted on a hemacytometer and adjusted to a final concentration of 5×10^4 CFU/mL. Inocula were loaded into flat-bottomed microtiter plates (Costar Corning, Lowell, MA, USA) and incubated at 37°C for 48 h. A no-growth end point was determined by eye. MIC testing was performed on RMLM isolates in triplicate, and a consensus mean was derived (median or mode). Susceptibilities of the aspergilloma isolates were determined once, except for 6 that were tested 3 times. Values of >8 mg/L were classed as 16.

Clinical or epidemiologic breakpoints/cutoffs have not been declared by the Clinical and Laboratory Standards Institute (CLSI) or EUCAST for azoles and *Aspergillus* spp. However, proposed epidemiologic cutoff values have been mooted for the latter (*22*), and we have recently proposed clinical breakpoints (*23*). Cutoffs used in this study were itraconazole and voriconazole >2 mg/L and posaconazole >0.5 mg/L (we have not defined an intermediate zone of susceptibility).

Sequencing

DNA was extracted by using commercially available kits (FastDNA Kit, Q-biogene, Cambridge, UK; Ultraclean Soil DNA Isolation Kit, MO BIO Laboratories Inc., Cambridge; and DNeasy plant tissue kit, QIAGEN, Crawley, UK). The entire coding region of the *cyp51A* gene was amplified as previously described (*7*), except 3 mmol/L $MgCl_2$ was used and both strands were sequenced using 8 primers (*7*). Twelve of the aspergilloma (A) isolates were sequenced with only 1 primer, covering the region of interest in this case. Sequences were aligned against the sequence from an azole-susceptible strain (GenBank accession no. AF338659), and mismatches were identified by using AlignX (VectorNTI; Invitrogen, Paisley, UK). Mutations were confirmed by repeating the PCR and sequencing both strands by using the closest 2 primers. Isolates with an alteration in the *cyp51A* gene at codon 98 were also investigated for promoter modifications by sequencing this region (*17*). GenBank accession numbers for the *cyp51A* sequences determined in this study are EU807919−EU807922 and FJ548859−FJ548890.

Microsatellite Typing

Six microsatellite loci (3A, 3B, 3C, 4A, 4B, 4C) were amplified as previously described (*24*). Initially some amplicons were sequenced, whereas later ones were sized by using capillary electrophoresis on an ABI PRISM 3130×l Genetic Analyzer (Applied Biosystems, Warrington, UK). Electrophoresis data were analyzed by using Peak Scanner Software version 1.0 (Applied Biosystems); amplicon sizes were adjusted by using a correction factor derived from sequenced alleles to determine the actual sizes of alleles (*25*). Concatenated multilocus microsatellite genotypes were created for each isolate and used to generate allele-sharing genetic distance matrices, D_{AS}. Here, $D_{AS} = 1 -$ (the total number of shared alleles at all loci / n), where n is the total number of loci compared (*26*). Subsequently, phylogenetic comparisons using 5 of the loci (not 3B) were performed with the software PAUP* 4.0 (www.paup.csit.fsu.edu) by using the neighbor-joining algorithm with the minimum-evolution option active. The strength of support for relationships was assessed by using 1,000 bootstrap resamples of the dataset.

Results

Susceptibility

The susceptibility of 519 *A. fumigatus* RMLM culture collection isolates was determined. All isolates were tested for susceptibility against itraconazole and amphotericin B; 456 and 118 isolates were also tested against voriconazole and posaconazole, respectively. Subsequently, all itraconazole-resistant isolates were tested against voriconazole and posaconazole. Geometric means, ranges, MIC_{50} (median MIC), and MIC_{90} (90% of the isolates tested had a MIC at or below this level) values are shown in Table 1. Amphotericin B susceptibility was retained in the 34 itraconazole-resistant isolates tested. Of these, 65% (22) were cross-resistant to voriconazole and 74% (25) were cross-resistant to posaconazole. We did not identify any isolates that were resistant to voriconazole or posaconazole while remaining susceptible to itraconazole.

Five percent of 400 isolates were resistant to itraconazole (when duplicate isolates from the same patient with similar susceptibility profiles were removed from the analysis). The overall frequency of itraconazole resistance in this collection (with repeat specimens included) was 7% (n = 519). The first case of azole resistance in this collection was seen in 1999. The frequency of resistance since 2004 (8%) has increased significantly (Fisher exact test, $p<0.001$), compared with the period prior to 2004 (Figure 1).

Azole Exposure in Patients with Azole-Resistant Isolates and Response to Therapy

Of the 17 patients identified for respective review, limited data were available for 3 patients. Of the remaining 14 patients with antifungal data (Table 2), azole exposure of 1–30 months before the identification of the first resistant isolate was evident for all except patient 7. Thirteen patients received itraconazole as initial therapy, and 12 of these were evaluable. Infections failed to respond to therapy in 7 itraconazole-treated patients (i.e., their disease progressed), although 3 patients appeared to improve before their conditions began to deteriorate. Infections failed to improve with azole therapy in 5 patients (i.e, their disease remained stable), and no patient had a sustained response to therapy. Nine of the 12 patients had at least 1 therapeutic concentration of itraconazole (>5.0 mg/L) documented at steady state during their treatment course (online expanded version of Table 2, available from www.cdc.gov/EID/content/15/7/1068-T2.htm). The infection in patient 1 (treated with voriconazole only for 18 months) failed therapy, and the 1 isolate identified had MICs of >8 mg/L for both itraconazole and voriconazole.

Of the 14 patients with available data, 2 had invasive disease; 9 had chronic diseases with ≥1 aspergillomas; 2 had allergic bronchopulmonary aspergillosis; and 1 had *Aspergillus* bronchitis. At least 5 of the patients died of progressive infection, despite alternative therapies for some.

Mutations in the *cyp51A* Gene

A summary of Cyp51A amino acid substitutions and azole cross-resistance patterns identified in 34 resistant isolates from our clinical culture collection is shown in Table 3 and listed by line in the online Appendix Table (available from www.cdc.gov/EID/content/15/7/1068-appT.htm). The sequences of all 5 azole-susceptible isolates examined were identical to that of a previously published *cyp51A* gene sequence from an azole-susceptible isolate (AF338659). No *cyp51A* mutations were found in 3 itraconazole-resistant isolates (from 2 patients). In addition to the L98H substitution, 2 isolates from 2 patients had a 34-bp sequence that was duplicated in the promoter region (*16,17*) of the *cyp51A* gene. One isolate had 2 amino acid substitutions, H147Y and G448S. Three isolates from 2 patients had the same 6 mutations, 3 nonsynonymous ones (F46Y, M172V, E427K), along with 3 synonymous (silent) alterations at codons 89, 358, and 454 (data not shown), and an isolate from a third patient had additional mutations (N248T, D255E) as well as these 6. Four novel mutations were found (H147Y, P216L, Y431C, and G434C). The isolate bearing the P216L mutation was resistant to itraconazole and posaconazole, where-

Table 1. MICs for 519 *Aspergillus fumigatus* isolates from RMLM culture collection, 1992–2007*

	Susceptibility results, mg/L							
	Itraconazole		Voriconazole		Posaconazole		Amphotericin B	
Isolate group (no. isolates)	GM (range)	MIC_{50}/ MIC_{90}	GM (range)	MIC_{50}/ MIC_{90}	GM (range)	MIC_{50}/ MIC_{90}	GM (range)	MIC_{50}/ MIC_{90}
RMLM collection (519), 1992–2007	0.46 (≤0.015–>8)	0.25/2	0.92 (0.125–>8)	1/2	0.22 (0.03–>8)	0.125/2	0.34 (0.06–2)	0.25/1
Azole resistant (34)	16.0 (>8)	>8/>8	3.69 (0.125–>8)	4/>8	1.70 (0.125–>8)	1/>8	0.22† (0.06–0.5)	0.25/0.5
Percentage resistant	100%		65%		74%		0%	
Aspergilloma (18)	16.0 (>8)	>8/>8	2.16 (0.5–4.0)	4/4	1.92 (0.125–>8)	1/>8	0.10‡ (0.06–0.125)	0.125/ 0.125

*RMLM, Regional Mycology Laboratory Manchester; GM, geometric mean. Values >8 mg/L were classed as 16 mg/L for GM analysis. See also the online Appendix Table, available from www.cdc.gov/EID/content/15/7/1068-appT.htm.
†n = 28.
‡n = 6.

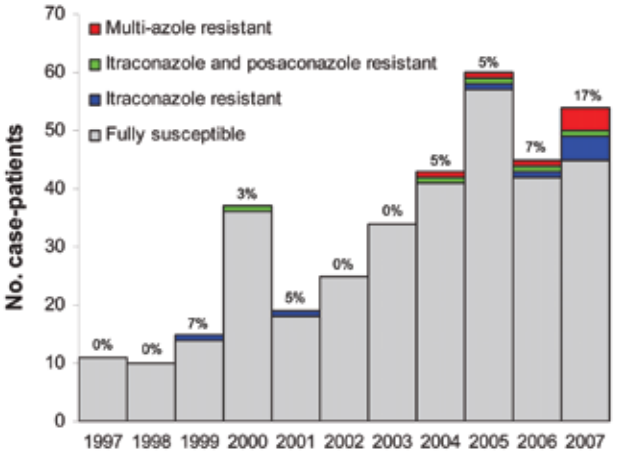

Figure 1. Azole resistance in clinical *Aspergillus fumigatus* isolates received in the Regional Mycology Laboratory Manchester, UK, 1997–2007. Overall azole resistance for each year is shown above each column as a percentage. Data do not include sequential isolates from the same patient.

as the isolates with Y431C and G434C showed pan-azole resistance phenotypes.

Patient 3 had 2 respiratory samples taken while she was alive, in addition to 18 aspergilloma isolates sampled at autopsy. All isolates were resistant to itraconazole (>8 mg/L), and 1 of 2 different mutations at codon 220 was detected in the *cyp51A* gene. Isolates with a methionine-to-lysine substitution were highly cross-resistant to voriconazole (4 mg/L) and posaconazole (>8 mg/L), whereas those with an alteration to threonine had variable voriconazole (0.5–4 mg/L) and posaconazole (0.125–1 mg/L) MICs.

Microsatellite Typing

The relatedness of isolates obtained from patients 3, 4, 5, 6, 8, 9, and 13 were compared by microsatellite typing (Figure 2). The isolates from 5 patients consisted of a susceptible/resistant pair, whereas an overlapping group of 4 patients had more than 1 *cyp51A* mutation. All isolates were from the lower respiratory tract, except the resistant isolate from patient 5, which was from a cerebral lesion.

Multiple isolates from 5 of 7 patients had identical or nearly identical genotypes. The isolates from 2 of these 5 patients (3 and 6) differed by 1 and 2 trinucleotide repeat units, respectively, at the most polymorphic locus (3A). Three matched sets (isolates pre- and postdevelopment of resistance) were identified, where resistance almost certainly evolved from an originally susceptible strain.

Figure 2 shows an unrooted tree of the phylogenetic relationships, derived from 5 of the 6 microsatellite markers, for the isolates from these 7 patients plus 18 *A. fumigatus* isolate controls. Only bootstrap values >90 are shown. Strains from these 7 patients are distributed among other clinical isolates; statistically supported clustering is not evident. Therefore, none of the azole-resistant isolates have been transmitted from patient to patient, indicating that they have all evolved independently from different original strains. The only statistically supported clades contain isolates that only differ from each other by 1 of the 5 markers.

Discussion

Itraconazole resistance and azole cross-resistance in *Aspergillus* spp. have been reported infrequently, which suggests that they are infrequent events to date. A contributing factor to this low prevalence has been variability in testing between laboratories. Since the initial report of resistance in isolates collected before the licensure of itraconazole, substantial improvements in susceptibility testing methods that allow confidence in reported azole MICs have been implemented. Recommended methods are now promulgated by the CLSI method M38-A2 (*27*) and EUCAST (*21*), and work is ongoing to establish internationally agreed interpretative cutoffs (*22*) and clinical breakpoints (*23*).

By using such methods, some researchers have documented and published the frequency of itraconazole resistance in clinical *A. fumigatus* isolates (*8,28–32*); frequency ranged between 2% and 6%. However, most of these studies included fewer isolates (<200) than our study (519) and covered the pre-2004 era. The frequency of itraconazole resistance in our collection before 2004 was 1%; since 2004, however, it has been remarkably high at 8%. The high frequency probably reflects, at least in part, the specialized referral base for patients with chronic and allergic aspergillosis at our center, although there has been no material change in catchment area in the past decade. Referral numbers are rising, however, and susceptibility testing of isolates of patients receiving therapy has been more frequent since 2003.

Another remarkable aspect of this study is the diversity of *cyp51A* mutations. Both previously published and novel alterations were identified in our resistant isolates (Table 3). In contrast, a recent series of 32 itraconazole-resistant isolates from the Netherlands was published; 94% had the same 2 alterations: an L98H-aa substitution in Cyp51A, in combination with a duplication in the promoter region (*32*). This combination of mutations was found in 2 of our isolates from 2 patients.

Several authors have identified hot-spot regions associated with resistance in clinical isolates at codons 54 (*6,10–13,22*), 98 (*16–18,22,32,33*), and 220 (*6,14,15,22,32*) in the *cyp51A* gene. We previously reported an alteration at codon 138 (G138C) in multiple isolates from 1 patient (*7*). A single clinical isolate with a mutation at codon 448 (G to S) has also been previously reported (*34*). In addition, G138R and G448S mutants have been generated in the

laboratory and were azole resistant (*35*). Mutations in codons 46, 172, 248, 255, and 427 have been found in azole-susceptible strains by us (A. Albarrag, unpub. data) and others (*22*) and so are not associated with resistance. The resistant isolates with these mutations must therefore have another resistance mechanism. Four novel *cyp51A*

Table 2. Clinical information for 14 patients with azole-resistant *Aspergillus fumigatus* infections*

Patient no.	Location	No. isolates	*Aspergillus* disease	Other diseases, y	Treatment, duration	Serum azole levels, mg/L†	Outcome/survival
1	Cambridge, UK	1	CCPA with aspergilloma	Breast cancer, 1990; *M. malmoense* pulmonary tuberculosis, 1999 and 2005	Vori 200–400 mg, 18 mo	ND	Clinical and radiologic failure/ alive
2	Copenhagen, Denmark	1	ABPA	CF, concomitant bacterial colonization with *Staphylococcus aureus* and *Achromobacter*	Itra 200 mg, 14 mo (plus previous courses)	ND	Unknown/alive
3	Manchester, UK	2‡	CCPA with aspergilloma CFPA	Pulmonary TB with residual bilateral UL scarring and LUL cavity, 1986; smoke inhalation, 1989	Itra 400 mg, 90 mo	15.0–26.0§	Clinical failure/died
4	Manchester, UK	3	CCPA with aspergilloma	COPD, squamous cell carcinoma with LUL segmentectomy, 1992	Itra 400 mg, >2 mo	2.9–11.3	No Improvement/died
5	Montreal, Quebec, Canada	2	Cerebral aspergillosis, 1998 Nov	AML-M2, 1997; RUL lobectomy, 1997; AlloHSCT, 1998; GVHD	Itra 400 mg, 4 mo	ND	Regression of cerebral abscess, IPA with respiratory failure/died
6	Manchester, UK	2	CCPA with aspergilloma	COPD, *M. szulgai* pulmonary infection, 2003; celiac disease	Itra 200–400 mg, 1 mo	<0.8 (200 mg), 5.3–7.7 (400 mg)	Clinical failure/died
7	Manchester, UK	1	Acute invasive pulmonary infection	COPD, possible bronchiectasis	Itra 600–400 mg, 1 mo; vori 400 mg; 12 d	17.0–21.0 (itra)	No improvement, switched to vori, developed toxicity/died without IPA
8	Northampton, UK	2	ABPA	Bronchiectasis, asthma, AVR, hypermobility syndrome; *M. xenopi* pulmonary infection, 2007	Itra 200–400 mg, 9 mo	0.0–5.2	Initial improvement, then failure/alive
9	Liverpool, UK	12	CCPA with bilateral aspergilloma, CFPA	Pulmonary sarcoidosis, 1988	Itra 200–400 mg, 30 mo	0.9–10.3	Clinical failure/died
10	Manchester, UK	2	*Aspergillus* bronchitis	Bronchiectasis, onychomycosis, 2007; α-1-antitrypsin deficiency	Itra 400 mg pulse, 3 mo	ND	Itra resistance identified, treated with posa/alive
11	Manchester, UK	2	CCPA with aspergilloma	RUL pneumonia, 2002	Itra 400 mg, 1.5 mo	20.0–>25.6	No improvement/alive
12	Manchester, UK (Malawi origin)	1	CCPA with 2 aspergilloma	Pulmonary TB, 1995; HIV positive, HAART	Itra 400 mg, 18 mo	2.5–8.4	Improvement then progression/alive
13	Preston, UK	4	CCPA with aspergilloma	COPD, bronchiectasis, *M. avium* pulmonary infection, 2002 and 2006	Itra 600 mg, 10 mo	2.6–4.5	Progression/alive
14	Birkenhead, UK	1	CCPA with LUL aspergilloma	Sarcoidosis, COPD, celiac disease; aspergilloma removed as part of left lung transplant, 2007¶	Itra 400 mg, 11 mo	13.8–17.8	Unchanged, switched to vori/unknown

*CCPA, chronic cavitary pulmonary aspergillosis; *M., Mycobacterium*; vori, voriconazole; ND, not determined; ABPA, allergic bronchopulmonary aspergillosis; CF, cystic fibrosis; itra, itraconazole; CFPA, chronic fibrosing pulmonary aspergillosis; TB, tuberculosis; UL, upper lobe; LUL, left upper lobe; COPD, chronic obstructive pulmonary disease; AML, acute myeloid leukemia; RUL, right upper lobe; AlloHSCT, allogeneic haematopoietic stem cell transplant; GVHD, graft versus host disease; IPA, invasive pulmonary aspergillosis; AVR, aortic valve replacement; posa, posaconazole; HAART, highly active antiretroviral therapy. An expanded version of this table, showing complete data on all 17 patients, is available online at (www.cdc.gov/EID/content/15/7/1068-T2.htm).
†Determined by bioassay (target range 5–15 mg/L).
‡Plus aspergilloma isolates studied, taken at autopsy.
§Received a generic formulation of itra, resulting in lower concentrations (i.e., 4.6 mg/L) and then probably was noncompliant at end of treatment period.
¶Successfully completed with vori treatment.

Table 3. Cyp51A amino acid substitutions and associated cross-resistance patterns in azole-resistant RMLM *Aspergillus fumigatus* isolates*

Cyp51A codon	No. patients	No. isolates	Amino acid substitutions	MIC, mg/L†		
				Itraconazole	Voriconazole	Posaconazole
F46‡	3	4‡	Y	>8	2–4	0.125–0.5
G54	4	5	E, R, V	>8	0.125–1	1–>8
L98+TR	2	2	H	>8	8	1–2
G138	1	10	C	>8	8–>8	2–>8
H147§	1	1§	Y	>8	>8	0.5
M172‡	3	4‡	V	>8	2–4	0.125–0.5
P216	1	1	L	>8	1	1
M220	3	4	K, T	>8	1–4	0.5–>8
N248‡	1	1	T	>8	2	0.25
D255‡	1	1	E	>8	2	0.25
E427‡	4	5‡	G, K	>8	2–4	0.125–0.5
Y431	1	1	C	>8	4	1
G434	1	1	C	>8	4	1
G448	2	2	S	>8	>8	0.5–1
No substitutions	2	3	NA	>8	2–8	0.25–1

*RMLM, Regional Mycology Laboratory Manchester; TR, tandem repeat in *cyp51A* promoter; NA, not applicable. Synonymous mutations not shown. Some mutations are associated with resistance but may not be causal (see text).
†Putative cut-off values for resistance are itraconazole and voriconazole >2 mg/L and posaconazole >0.5 mg/L.
‡F46Y found with M172V and E427K in 4 isolates along with 3 silent mutations. E427G seen alone in 1 isolate. N248 and D255 found in combination with 46/172/427 in 1 isolate.
§Found with G448S in 1 of 2 isolates.

mutations, 3 of which were unassociated with any other mutations (in codons 147, 216, 431, and 434), were identified in this series, although their association with resistance remains to be confirmed experimentally. The H147Y substitution is probably unimportant for resistance because it was found with G448S in 1 isolate and the cross-resistance profile of this isolate was identical to an isolate that had only G448S. We did not find any examples of previously reported mutations at codons 297 and 495 (*17,32*) or 22, 394, 491, and 440 (*14*) in our collection. Three of our resistant isolates had no mutations in their *cyp51A* gene, indicating the presence of other resistance mechanisms.

The position and type of amino acid substitution within the Cyp51A protein determines the pattern of azole cross-resistance (Table 3), which is consistent with predicted structural properties of the demethylase enzyme and its interaction with chemically different azole drugs (*36*). Resistance to itraconazole is usually associated with a reduction in posaconazole susceptibility, predictably because the 2 drugs are structurally similar; they have variably elevated posaconazole MICs compared with wild type isolates (*22,30*). Many of the isolates reported here reflect this MIC shift. Isolates with alterations at codons 98 (including the duplication in the promoter region), 138, 431, and 434 demonstrated cross-resistance to voriconazole and posaconazole. All isolates with substitutions at codons 54 and 216 remained susceptible to voriconazole. Some isolates in this study showed cross-resistance between itraconazole and voriconazole and not posaconazole, unlike the results in previous reports (*22,32*). However, this cross-resistance could be because of differing breakpoints; therefore, determination of an internationally agreed cutoff for posaconazole will be necessary to guide clinicians. No difference in amphotericin B MICs was seen in our azole-resistant isolates compared with susceptible ones, although the clinical utility of MIC testing of amphotericin B in *Aspergillus* spp. is suboptimal.

More than 1 azole-resistant *A. fumigatus* isolate was obtained from 6 of the 17 patients described. Microsatellite typing demonstrated that the isolates from each patient had evolved from a single original strain, because they were either identical at 6 markers or differed only in the most polymorphic marker. The resistant isolates from 4 patients had different *cyp51A* mutations. Given that a single colony is picked from the primary isolation plate and referred for susceptibility testing, additional mutants may have been found had multiple colonies been tested. Within this dataset, the chance of 2 isolates being identical by chance alone within a recombining population are infinitesimal given the high allelic variability that we observed (mean number of alleles per locus = 14; p value recovering the same multilocus genotype twice $\approx 14^5$). The existence of susceptible and resistant isolates that are genetically identical from 3 patients, and the phylogeny performed on the multiple-resistant isolates from an additional 4 patients, almost certainly indicates that the evolution of azole resistance has occurred in these patients independently and repeatedly from unrelated strains. The presence of genetically identical isolates with different *cyp51A* codon mutations in 3 patients (and 1 almost identical) suggests that they must have evolved independently from the same original strain, because the resistance mutations are not being accumulated sequentially

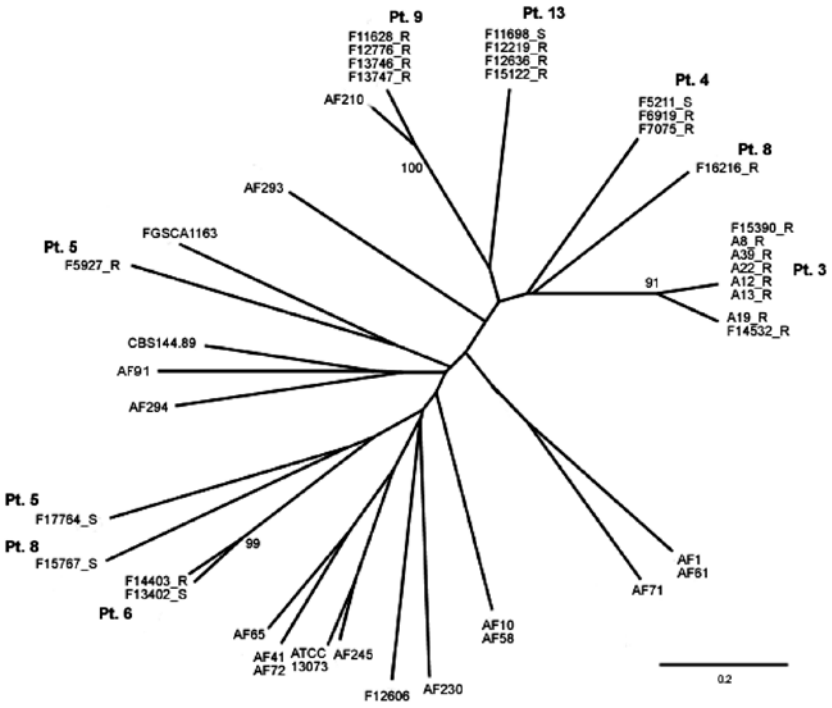

Figure 2. Unrooted phylogenetic tree showing the genetic relationship of isolates from 7 patients. The genetic relationship of these isolates is shown in relation to each other and to 18 other isolates. AF numbers belong to a collection of >200 isolates, held in Manchester, UK. ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; FGSC, Fungal Genetics Stock Center. Bootstrap values >90 only are shown. Scale bar indicates nucleotide substitutions per site.

as has been shown to happen in *Candida albicans* (*37*). The isolates from 2 patients had differing numbers of repeats of microsatellite marker 3A, which is further proof that strains are evolving in the lung. In contrast, Snelders et al. (*32*) suggested that many of their patients were infected with a primary resistant strain from the environment.

The referral base for these isolates includes a specialized clinical service for the management of aspergillosis. Many of our resistant isolates came from this group, in particular from 9 patients with chronic cavitary pulmonary aspergillosis with ≥1 aspergillomas, which may explain the high frequency of resistance in our center. Because surgery is not an option for most patients with chronic cavitary pulmonary aspergillosis, these patients usually require long-term (if not lifelong) antifungal therapy, under which conditions as we have shown, strains of *A. fumigatus* may evolve resistance. Another contributory explanation could be our systematic application of susceptibility testing of *Aspergillus* spp. isolates in all cases in which treatment is to be given.

In 6 of 10 patients, steady state itraconazole plasma level data were at or above minimum therapeutic levels (i.e., <5 mg/L), as determined by bioassay (*38,39*). Low plasma levels of itraconazole were attributable to limited bioavailability in some patients, low doses (i.e., 200 mg daily, the standard UK registered dose), drug interactions in patients with concomitant atypical mycobacterial infection, and use of generic itraconazole (*40*). Low plasma levels of itraconazole, in combination with the high proportion of patients in this study with prior azole exposure (13 out of 14), indicates that resistance primarily emerged during or after azole therapy.

Our observations are of concern on several fronts. We found a sudden rise in the frequency of azole resistance in *A. fumigatus* since 2004, and many isolates showed cross-resistance between all the currently licensed azole options. Clinical data indicate that resistance has occurred during and after azole therapy in all but 1 of these cases. The infections caused by azole-resistant isolates fail therapy or at best do not respond. The molecular epidemiology shows that resistance evolved in infecting strains within the lung, rather than by superinfection with a resistant stain from the environment. Because azoles are the only useful class of oral drugs for aspergillosis (and many other serious filamentous fungal infections), clinical management of these chronically infected cases is therefore problematic. Vigilance is called for to identify azole-resistant aspergilli, and novel classes of oral antifungal would be welcome for those infected with azole-resistant strains.

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Co-infections with Chikungunya Virus and Dengue Virus in Delhi, India

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Aedes aegypti mosquitoes are common vectors for dengue virus and chikungunya virus. In areas where both viruses cocirculate, they can be transmitted together. During a dengue outbreak in Delhi in 2006, 17 of 69 serum samples were positive for chikungunya virus by reverse transcription–PCR; 6 samples were positive for both viruses.

Chikungunya virus (CHIKV) was isolated in Tanganyika (now Tanzania) in 1953 (*1*). In Asia, this virus is transmitted almost exclusively by *Aedes aegypti* mosquitoes. India had its first CHIKV outbreak in 1963; it was followed by epidemics in other parts of the country (*2*). Recently, massive outbreaks of CHIKV have been reported from many islands in the Indian Ocean (*3*). Chikungunya outbreaks in India were reported in 2005, and 1.4 million chikungunya cases were reported from different states (*3*).

Estimated annual incidence of disease caused by dengue virus (DENV) is 50–100 million cases of dengue fever and 250,000 cases of dengue hemorrhagic fever; mortality rate is 25,000 per year in tropical and subtropical countries. Like CHIKV, DENV is also transmitted by *Ae. aegypti* and is endemic to urban and semiurban areas of India (*4*).

In Asia, the CHIKV-affected areas overlap with DENV-endemic areas (*5*,*6*) and provide opportunities for mosquitoes to become infected with both viruses. Co-infection with 2 dengue viruses (DENV-1 and DENV-4) was reported in Puerto Rico in 1982 (*7*). Since then, many cases of concurrent infections with multiple DENV serotypes have been reported in many countries. Since 2005, co-infections with >2 DENV serotypes have been reported in Delhi, India (*8*). Co-infections with DENV and CHIKV were reported in Calcutta, India, in 1967 (*5*). Subsequent serologic investigations in southern India indicated that the 2 viruses can coexist in the same host (*9*). We report detection by reverse transcription–PCR of co-infections with CHIKV and DENV in clinical samples obtained during the 2006 dengue outbreak in Delhi, India.

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The Study

Acute-phase blood samples were collected from 69 patients with clinically suspected cases of DENV/CHIKV co-infection. Viral RNA was extracted from serum samples by using the MagNA Pure Compact Nucleic Acid Isolation System (Roche Diagnostics, Basel, Switzerland). Published primers and cycling conditions were used for the amplification of DENV (*10*) and CHIKV (*11*). cDNA was synthesized by using avian myeloblastosis virus reverse transcriptase (Promega Corp., Madison, WI, USA) and downstream consensus primer (D2) for DENV and random hexamers for CHIKV. DENV typing was performed by using second-round amplification with type-specific primers (*10*). The amplified products were visualized by electrophoresis on 2% agarose gels. Because samples were received during a dengue outbreak, ethical clearance was not required.

Partial nucleotide sequences of the envelope 1 (E1) gene (294 bp) of CHIKV were determined by using an automated 310 DNA sequencer (Applied Biosystems Inc., Foster City, CA, USA). Sequences were aligned, analyzed, subjected to homology search by BLAST analysis (www.ncbi.nlm.nih.gov/Education/BLASTinfo/information3.html), and submitted to GenBank (accession nos. EU727159–63 and EF539265). Phylogenetic analysis of CHIKV sequences (Table) was conducted by using ClustalW (www.ebi.ac.uk/Tools/clustalw2/index.html) and MEGA version 3.1 software (*12*), Kimura 2-parameter distances, and neighbor-joining algorithms.

Of 69 samples tested, DENV RNA was detected in 48 and CHIKV RNA in 17. Of the 17 CHIKV-positive samples, 6 were co-infected with DENVs. Three of the 6 samples from patients co-infected with CHIKV/DENV contained DENV-3; 1 contained DENV-4, and 2 contained 2 DENV serotypes (1 contained DENV-3 and DENV-4 and 1 contained DENV-3 and DENV-1) (Figure 1).

Of the 17 CHIKV-positive patients, 10 were male and 6 were female. Information regarding age, sex, and clinical features was not available for 1 patient. Thirteen samples were from adults (>12 years of age) and 3 were from children (≤12 years of age).

Retrospective analysis of medical records identified clinical information for 6 patients co-infected with DENV and CHIKV. All 6 patients had fever, headache, joint pain, and low thrombocyte counts (<100,000/mm^3). The patients with only CHIKV infection had fever, headache, and joint pain. Of the 6 patients with co-infections, 2 had dengue hemorrhagic fever with central nervous system (CNS) involvement. CNS involvement and hemorrhagic manifestations may be caused by DENVs because these manifestations are common in patients infected with DENV; CNS involvement has been documented in persons with DENV infections (*13*). In 2 patients with CNS involvement, 1 was

infected with DENV-3 and 1 was infected with DENV-4. Of the 6 patients with co-infections, 5 fully recovered and 1 died.

Phylogenetic analysis of partial E1 gene sequences demonstrated that all CHIKV strains from Delhi grouped with isolates obtained during 2006 from southern India and islands in the Indian Ocean and belonged to the Central/East African genotype (Figure 2). This finding indicates that during 2006 similar strains were circulating throughout India. Isolates obtained in India during 1963–1973 clustered with isolates from Thailand (Thai 62–78) and formed a separate cluster in the Asian genotype.

Table. Chikungunya virus sequences, including strains from Delhi and southern India, used for phylogenetic analysis*

Sequence no.	Laboratory ID or isolate name	Year	State/country	GenBank accession no.
1	GOA 018	2006	Goa/India	EF187902
2	HYD 349	2006	Hyderabad/India	EF187893
3	GWL 008	2006	Madhya Pradesh/India	EF187904
4	HYR023	2006	Karnataka/India	EF187899
5	CHTR 54	2006	Andhra Pradesh/India	EF187897
6	IND06 AP5	2006	Andhra Pradesh/India	DQ520744
7	IndKL 01	2006	Kerala/India	EU119154
8	IND06 MH1	2006	Maharashtra/India	DQ520734
9	IND06 AP6	2006	Andhra Pradesh/India	DQ520745
10	IND06 MS2	2006	Andhra Pradesh/India	DQ520740
11	IND06 MS1	2006	Andhra Pradesh/India	DQ520741
12	IND06 KA3	2006	Karnataka/India	DQ520738
13	PON1	2006	Pondicherry/India	EF113095
14	IND05 KA1	2005	Karnataka/India	DQ520737
15	REUNION	2006	Réunion Island	DQ443544
16	IND06 AP3	2006	Andhra Pradesh/India	EF027134
17	IND06 MH2	2006	Maharashtra/India	EF027136
18	IND06 MH3	2006	Maharashtra/India	DQ520736
19	ROSS	1953	Tanzania	AF490259
20	TAN53	1953	Tanzania	AF192905
21	IND00 MH4	2000	Maharashtra/India	EF027139
22	CONGO02	2000	Congo	AY549581
23	CONGO03	2000	Congo	AY549579
24	CONGO01	2000	Congo	AY549583
25	S27AFRICA	1953	Tanzania	NC004162
26	MALAYA98A	1998	Malaysia	AF394210
27	MALAYA98B	1998	Malaysia	AF394211
28	THAI95	1995	Thailand	AF192897
29	THAI96	1996	Thailand	AF192900
30	THAI88	1988	Thailand	AF192896
31	PHILLIP85	1985	The Philippines	AF192895
32	INDON85	1985	Indonesia	AF192894
33	THAI75	1975	Thailand	AF192898
34	THAI78	1978	Thailand	AF192899
35	THAI62	1962	Thailand	AF192908
36	IND71CH1	1971	Tamil Nadu/India	DQ520751
37	IND63WB1	1963	West Bengal/India	EF027140
38	IND64CH2	1964	Tamil Nadu/India	DQ520748
39	SENEG66	1966	Senegal	AF192891
40	NIGER64	1963	Nigeria	AF192893
41	SENEG83A	1983	Senegal	AY726732
42	SENEG83B	1983	Senegal	AF192892
43	O'NYONG-NYONG	1996	Uganda	AF079456
44	DEL/1467/06	2006	Delhi/India	EF539265
45	DEL/758/06	2006	Delhi/India	EU727160
46	DEL/868/06	2006	Delhi/India	EU727163
47	DEL/968/06	2006	Delhi/India	EU727161
48	DEL/1307/06	2006	Delhi/India	EU727162
49	DEL/1795/06	2006	Delhi/India	EU727159

*ID, identification number.

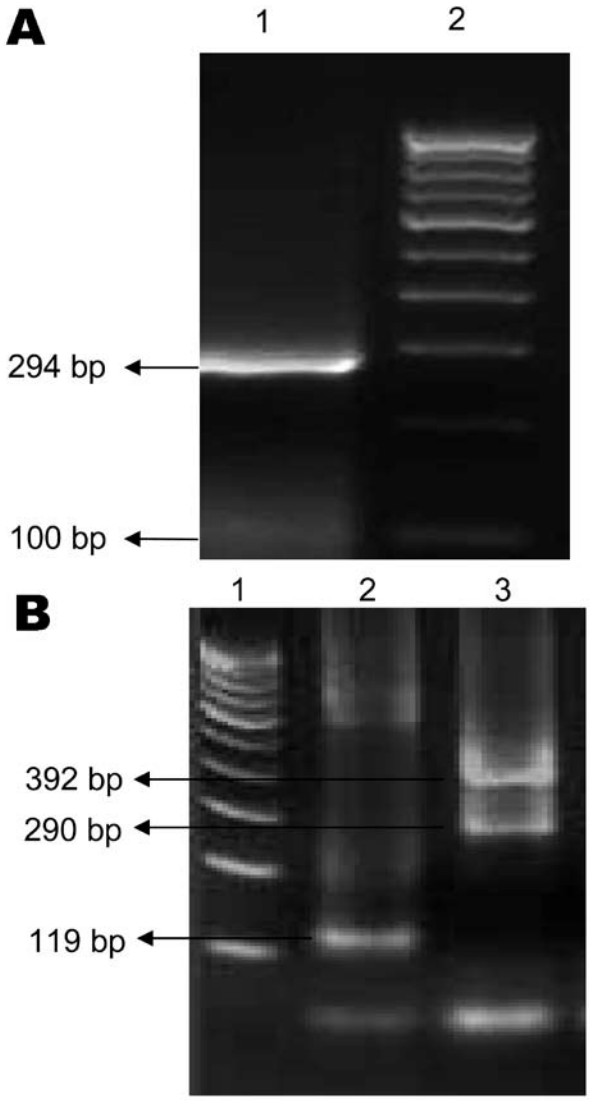

Figure 1. Agarose gel electrophoresis showing chikungunya virus (A) and dengue virus (B) PCR products. A) Lane 1, 294-bp product specific for chikungunya virus; lane 2, 100-bp DNA marker. B) Lane 1, 100-bp DNA marker; lane 2, 119-bp product specific for dengue 2 virus; lane 3, 290-bp product for dengue 3 virus and 392-bp product for dengue 4 virus.

Conclusions

For many years, it appeared that CHIKV had disappeared from India, but late in 2005 the virus reemerged on Reunion Island and in India (*3*). Confirmed cases of CHIKV infection have been reported from Delhi, Haryana, Uttar Pradesh, and Rajasthan provinces in northern India, although these states did not have large-scale epidemics (*14*).

DENV infections are endemic to northern India; in recent years, increasing trends of cocirculation of multiple DENV serotypes in Delhi suggest that DENVs are becoming hyperendemic to this region (*8*). During 2006, DENV and CHIKV were detected in Delhi (*14*). Because the clinical features of DENV and CHIKV are similar, CHIKV infections may go undiagnosed in DENV-endemic areas. In India, *Ae. aegypti* mosquitoes are primary vectors for DENV and CHIKV, and opportunities for co-infections in humans are increased by the feeding behavior of the mosquito (*15*), low socioeconomic conditions, and high population density.

We report co-infections with DENV and CHIKV in India after a long absence of the viruses in this region. It is difficult to comment on increased severity of illness in patients with DENV/CHIKV co-infections because the num-

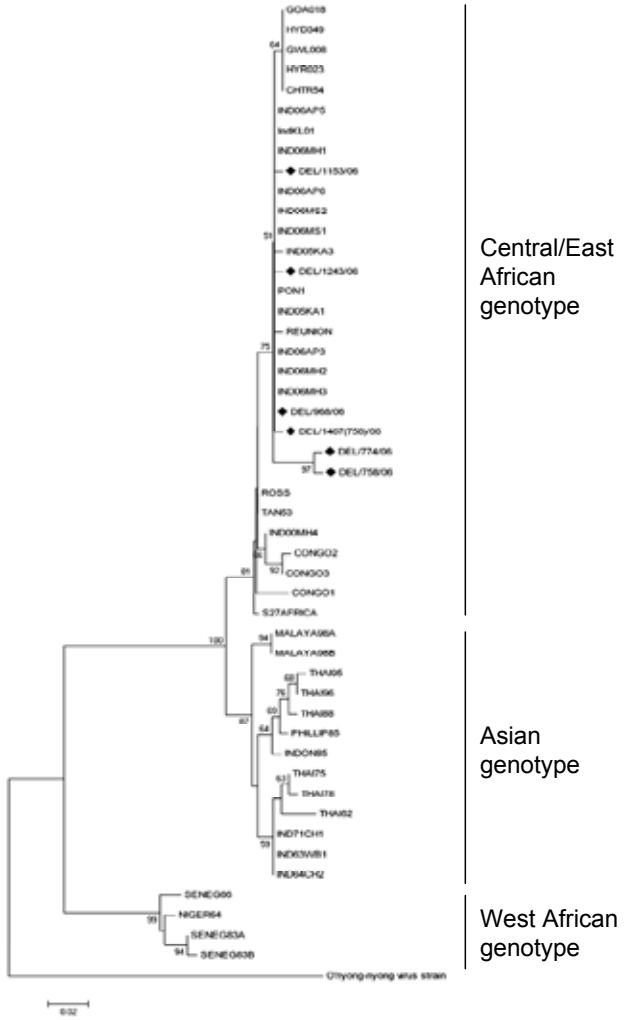

Figure 2. Phylogenetic analysis of partial envelop 1 (E1) gene sequences (294 bp) of chikungunya virus strains from the 2006 dengue outbreak in Delhi, India. Neighbor-joining tree was constructed by using E1 gene sequences from various chikungunya virus sequences. O'nyong-nyong virus (AF079456) was used as an outgroup. Percentage bootstrap support is indicated by the values at each node. Delhi strains are indicated by a diamond. Scale bar indicates nucleotide substitutions per site.

ber of patients tested was small. Additional clinical information is needed to determine the influence of co-infections on clinical expression of dengue and chikungunya fever.

Our study indicates that co-infections with CHIKV and DENV occur in areas where these 2 viruses cocirculate. Concurrent infections may result in illness with overlapping signs and symptoms, making diagnosis and treatment difficult for physicians. Repeated outbreaks of dengue, recent activity of CHIKV, and CHIKV/DENV co-infections in the Delhi area suggest that the epidemiology of these viruses is changing in this region and that these viruses are becoming endemic to this region. Thus, in clinically suspected cases of dengue or chikungunya fever, it is advisable to test for both viruses in areas where they cocirculate.

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Influenza Virus A (H1N1) in Giant Anteaters (*Myrmecophaga tridactyla*)

Sally Nofs,[1] Mohamed Abd-Eldaim,[1] Kathy V. Thomas, David Toplon, Dawn Rouse, and Melissa Kennedy

In February 2007, an outbreak of respiratory disease occurred in a group of giant anteaters (*Myrmecophaga tridactyla*) at the Nashville Zoo. Isolates from 2 affected animals were identified in March 2007 as a type A influenza virus related to human influenza subtype H1N1.

Emergence of viruses in new hosts is a continuing concern in public health surveillance and thus is the focus of intense research. The ability to move among different species may enable mutations and changes in phenotypes as the virus adapts to a new host (*1*). Influenza virus is a prime example of a pathogen with the ability to infect not only its avian reservoirs but also mammalian species such as swine, horses, dogs, cats, ferrets, whales, and humans.

In March 2007, we documented the occurrence of influenza in giant anteaters (*Myrmecophaga tridactyla*). Giant anteaters are indigenous to neotropical regions of Central and South America, and, although they are extinct or endangered in some regions, overall their status is listed as near threatened by the International Union for Conservation of Nature (*2,3*).

The Study

A colony of 11 adult anteaters (7 males, 4 females) and 1 neonate was housed at the Nashville Zoo at Grassmere, Nashville, Tennessee, USA. The colony experienced an outbreak of respiratory disease beginning in February 2007. The anteaters were housed separately in stalls in the same building with shared ventilation, with the exception of the nursing neonate who was housed with his dam. There was no contact with animals outside the colony. The primary caretaker of the colony had no contact with other animals housed at the zoo. No other species experienced respiratory disease at the zoo during the outbreak. Only the primary caretaker had sustained direct contact with any members of the colony.

The index case occurred on February 8 in 1 animal that was being treated twice a day for a superficial wound. The respiratory disease was characterized clinically by severe nasal discharge and congestion, inappetence, and lethargy. Within several days, all adult animals in the colony were affected. Only the neonate appeared to remain unaffected. The caretakers overseeing the colony, with the exception of the attending veterinarian, were also ill with respiratory disease, including the primary caretaker. The onset of the caretakers' illness coincided with the illness in the anteaters. No diagnostic testing was conducted for the caretakers during the outbreak.

Nasal discharge samples were collected on February 15 for virus isolation from 3 animals, including the index case. These samples were shipped overnight on cold packs to the Clinical Virology Laboratory at the University of Tennessee College of Veterinary Medicine. These samples were prepared for virus isolation in Dulbecco minimal essential medium supplemented with antimicrobial drugs, antifungals, and 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA; and Cambrex Bioscience Walkersville, Walkersville, MD, USA). Cell lines injected included Vero, rabbit kidney, MDCK, and Crandell-Reese feline kidney (American Type Culture Collection, Manassas, VA, USA). Cytopathic effects were noted in the MDCK cell lines on first passage in 2 of the 3 isolations; no cytopathic effects were noted in the isolation from the third sample.

Cell culture supernatant from the infected flasks was tested for hemagglutination by using 10% guinea pig erythrocytes, and agglutination was observed. Virus-infected cell pellets were prepared for electron microscopic examination (*4*). Examination showed an enveloped virus of 100–120 nm diameter (www.vet.utk.edu/pubs/mkenned2/EID_08-1574). Slides of infected cells were also prepared and examined by direct immunofluorescent antibody (IFA) assay by using influenza A- and B-specific antiserum (Diagnostic Hybrids, Athens, OH, USA); positive fluorescence was observed only with type A-specific antiserum. Based on the morphology and IFA results identifying a type A influenza virus, reverse transcription and PCR were conducted on the isolate from one of the animals by using type A-specific primers encompassing the entire hemagglutinin (HA), neuraminidase (NA), nonstructural protein 1 (NS1), and RNA polymerase 2 (PB2) gene segments as previously described (*5*). Nucleotide sequencing of HA, NA, PB2, and NS1 gene amplification products were conducted by Molecular Biology Resources (University of Tennessee, Knoxville, TN, USA) using an ABI prism dye terminator cycle

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sequencing reaction kit and an ABI 373 DNA sequencer (Perkin Elmer, Foster City, CA, USA) (Genbank accession nos. EU543278, EU543279, FJ478393, and FJ785200, respectively). High nucleotide identity (≥99%) was found between the anteater isolate and human influenza virus isolate type A strain Tennessee/UR06-0119/2007(H1N1) and others from the United States isolated in 2006 and 2007 (data not shown). Phylogenetic analysis (MegAlign program with ClustalW align, Lasergene package; DNASTar, Madison, WI, USA) of the HA and NA amplification products indicated a close relationship among these isolates (Figure).

Serologic analysis was not feasible at the time of the outbreak due to the need to use anesthesia to immobilize the animals for blood collection. However, samples collected from 3 animals in months following the outbreak were made available for serologic testing, as well as samples from these same animals collected at various times prior to the outbreak. The samples had been stored at –20°C. Testing was conducted by hemagglutination inhibition using the anteater influenza isolate (*6*). The serologic results are shown in the Table. Evidence of seroconversion was observed in 2 of the animals, and 1 animal had evidence of infection prior to the 2007 outbreak.

Conclusions

This respiratory disease outbreak among members of an anteater colony was caused by an influenza virus isolate closely related to the human influenza virus (H1N1) strains circulating in the concurrent year. Serologic analysis of samples from animals involved in the outbreak confirmed infection; seroconversion was documented in 2 animals, and 1 animal appeared to have been exposed to and infected with influenza virus a year before the described outbreak. This animal had been imported as a juvenile in 2003 and was ill at that time with a respiratory disease. It is not known what the cause of this earlier illness was or if exposure to influenza in the intervening years had occurred. Analysis of 4 viral genes sequenced indicated high homology with the human influenza virus (H1N1) isolates.

The differences between the anteater isolate and circulating human strains did not occur at sites known to be antigenically or functionally important; thus, these minor changes do not appear to alter the antigenicity or the function of the encoded proteins (*7*, E. Gorvokova, pers. comm.). We concluded that, based on the genetic sequence of the virus isolated from the anteaters and on the fact that the colony was not exposed to animals other than the human caretakers, the caretakers were the most likely source of the virus affecting the anteater colony. Further genetic sequencing will be required to determine if this interspecies transmission arose as a result of mutations of the virus, including reassortment.

Influenza is known to cross species lines into mammalian species. Birds, in particular waterfowl (Family Anatidae), are the natural reservoir for influenza viruses. Reassortment between avian and mammalian strains may occur in swine because they express receptors for avian and mammalian influenza (*8*). An entire influenza virus may undergo interspecies transmission without reassortment. Two recent notable incidences of this are the transmission of equine influenza virus (H3N8) to dogs (*9*) and the transmission of avian influenza virus (H5N1) to humans as well as to various carnivore species (*10–13*). With the first men-

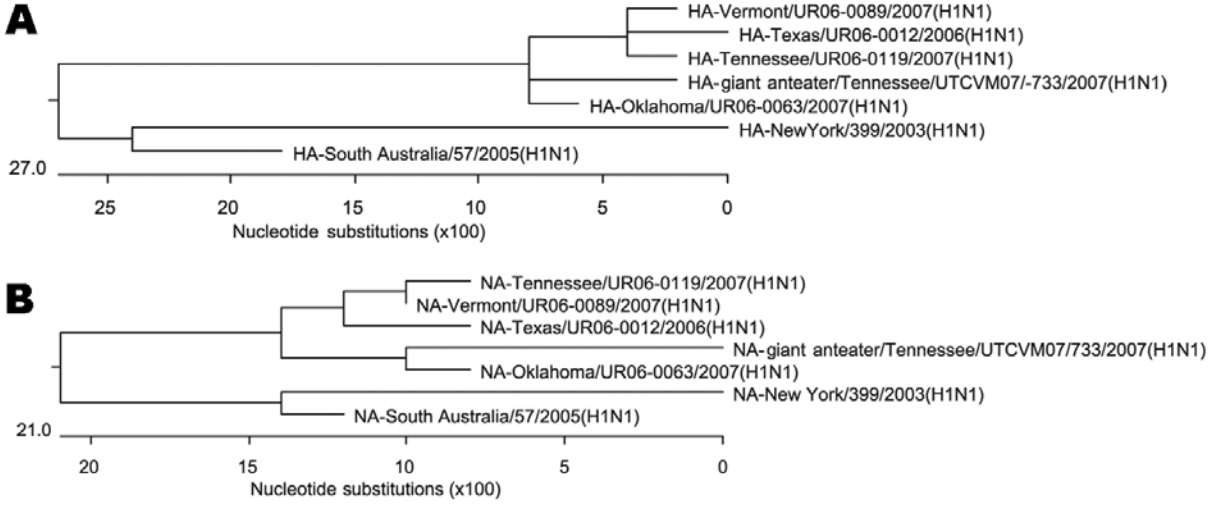

Figure. Phylogenetic tree based on the nucleotide sequence of the A) hemagglutinin (HA) gene and B) neuraminidase (NA) gene of the anteater isolate of influenza virus with 6 related isolates obtained from GenBank. GenBank accession numbers for the other isolates used: Tennessee/UR06-0119/2007(H1N1): HA-CY027379, NA-CY027381; Texas/ur06/0012/2006(H1N1): HA-CY025213, NA-CY025215; Vermont/UR06/0089/2007(H1N1): HA-CY025795; NA-CY025797; Oklahoma/UR06/0063/2007(H1N1): HA-CY027771, NA-CY027773; New York/399/2003(H1N1): HA-CY002808, NA-CY002810; and South Australia/67/2005(H1N1): HA-CY016691, NA-CY016693.

Table. Serologic results on samples collected from 3 anteaters before and after the February 2007 respiratory disease outbreak at the Nashville Zoo at Grassmere, Nashville, Tennessee, USA*

Animal no.	Before outbreak		After outbreak		Virus isolation
	Date collected	HI titer	Date collected	HI titer	
1	2004 Apr	0	2007 Aug	32	No sample
2	2006 Aug	4	2008 Jun	64	No sample
3	2006 Mar	32	2008 Oct	64	Virus isolated

*HI, hemagglutination inhibition.

tioned, efficient dog-to-dog transmission was documented. For avian influenza virus (H5N1), sustained human-to-human transmission has not occurred. It is not clear whether animal-to-animal spread of this influenza virus occurred within the anteater colony or whether all affected animals were infected directly from the caretakers.

The isolation of influenza virus in giant anteaters highlights the difficulty of influenza surveillance as the full spectrum of mammalian hosts for influenza virus remains unknown. This host variability could potentially impact human populations as possible sources of zoonotic spread of influenza.

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Intergenogroup Recombinant Sapovirus in Japan, 2007–2008

Wisoot Chanit, Aksara Thongprachum, Pattara Khamrin, Shoko Okitsu, Masashi Mizuguchi, and Hiroshi Ushijima

We investigated the incidence of sapovirus (SaV)–associated gastroenteritis in infants and children in Japan during 2007–2008 and characterized the diversity of SaV-positive strains. SaV was detected in 19 (4%) of 477 fecal specimens. The leading genogroup (79%, 15 cases) comprised intergenogroup recombinant SaVs (GII/GIV).

Sapovirus (SaV) is now considered a notable global enteropathogen of acute gastroenteritis in persons of all ages (*1*–*3*). As a member of the family *Caliciviridae*, SaV has a single-stranded positive-sense RNA genome of ≈7.3–7.5 kb that contains either 2 or 3 main open reading frames (ORFs 1–3). SaV ORF1 encodes for nonstructural proteins and the major capsid protein (VP1), and ORF2 (VP2) and ORF3 encode proteins of yet unknown functions. On the basis of VP1 nucleotide sequences, SaVs are divided into 5 genogroups (GI–GV), of which GI, GII, GIV, and GV strains are known to infect humans; SaV GIII infects porcine species. We investigated the incidence of SaV-associated gastroenteritis in infants and children in Japan during 2007–2008 and characterized the diversity of SaV-positive strains.

The Study

We collected 477 fecal specimens from nonhospitalized children with acute gastroenteritis in pediatric clinics in 5 localities in Japan (Tokyo, Sapporo, Saga, Osaka, and Maizuru) during July 2007–June 2008. Of these, 14 specimens were from Tokyo, 30 from Sapporo, 77 from Saga, 91 from Osaka, and 265 from Maizuru. We defined diarrhea as at least 3 passages of unformed (loose and watery) feces a day. We defined acute gastroenteritis as diarrhea and other symptoms, such as vomiting, fever, and abdominal pain. Children studied ranged in age from 1 month to 14 years (median 25 months). The Ethical Committees of The University of Tokyo and Aino University approved the study. A parent or guardian of each child provided informed consent.

RNA was extracted and purified by using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. Reverse transcription (RT) was performed with 5 μL of RNA template by using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). By using multiplex RT-PCR, 2 groups of diarrheal viruses were identified: 1) astrovirus, norovirus, and sapovirus and 2) rotavirus and adenovirus (*2*). The PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized by staining with SYBR Safe (Invitrogen). Nucleotide sequences were determined by using a Big-Dye terminator cycle sequencing kit and ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The nucleotide sequences were aligned by using ClustalX (http://bips.u-strasbg.fr/fr/Documentation/ClustalX), and distances were calculated by using the 2-parameter Kimura method. Phylogenetic trees with bootstrap analysis from 1,000 replicas were generated by using the neighbor-joining method. The sequences of SaV strains detected in the study have been submitted to GenBank under accession nos. FJ445092–FJ445110.

Of the viruses isolated from diarrhea samples, group A rotavirus was the most prevalent (20.5%), followed by norovirus (19.3%), adenovirus (4.4%,) sapovirus (3.9%), group C rotavirus (0.8%), and astrovirus (0.2%). In addition, we found viral mixed infections in 1.8%.

Sapovirus was detected in 19 (4%) fecal specimens. The highest incidence of SaV infection was in the 1-year-old group (9 [47%]), and most (13 [68%]) of these infections occurred in infants and children <3 years of age. Infections increased slightly during December through February (12 cases). The most common signs and symptoms in SaV-infected children were diarrhea (19 children [100%]), fever >100°F (5 [26%]), and vomiting ≥3 times a day (3 [15%]).

Nineteen SaV sequences were analyzed by phylogenetic analysis and grouped by using the recent SaV capsid region classification scheme (*4*). Most SaV sequences belonged to genogroup IV (15 cases [79%]), followed by GI/4 (3 cases [16%]), and GI/1 (1 case [5%]). Three sequences of GI/4 genotype had 98%–100% nucleotide identity with each other and grouped with the Karachi/872/91/PK and Osaka/5836/JP strains known to belong to GI/4 genotype. One GI/1 sequence had 97% nucleotide identity and clustered with Manchester sequence (Figure 1).

Nucleotide sequence comparison of the identified 15 GIV shared little or almost no divergence among themselves (98%–100% identity), even when they were detected in regions of Japan distant from each other. They are likely to represent a single strain, 8208/Maizuru/08/JP. The 8208/Maizuru/08/JP sequence closely matched

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Ehime1107 and SW278 (*5*), and Yak2 (*6*) sequences, which were previously established as intergenogroup recombinant SaV strains with the GII polymerase region and GIV capsid region, with 97% and 96% nucleotide identities, respectively. To determine whether our GIV strains were the recombinant SaV, 5 of the 15 GIV strains were randomly selected as representative, and long genomic fragments that included part of the RNA polymerase and part of the capsid genes were amplified by using primers SR80/2 (5′-TGGGATTCTACACAAAACCC-3′) and SLV5749 (5′-CGGRCYTCAAA VSTACCBCCCCA-3′), which generated a 1,151-bp product. The products were directly sequenced, and capsid- and polymerase-based phylogenetic trees confirmed these strains as the recombinant SaVs (Figure 2). We suggest the GIV strains isolated in our study were intergenogroup recombinants. Also, these recombinant strains were detected in 4 locations distant from each other: Maizuru city (10 cases), Sapporo and Saga (2 cases each), and Osaka (1 case), which suggests that the recombinant strains were widely spread through the country.

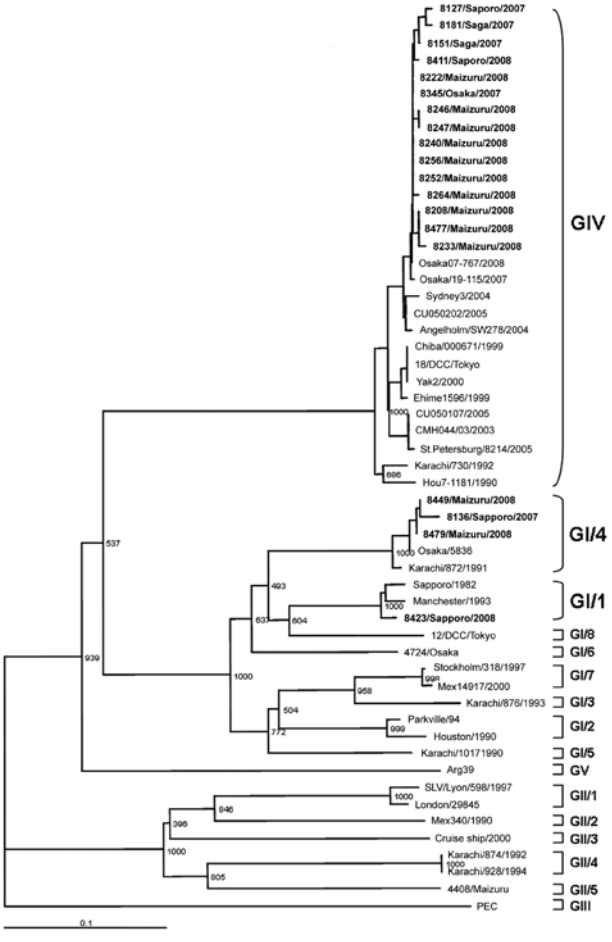

Figure 1. Phylogenetic tree of nucleotide sequences of sapovirus (SaV) strains (shown in **boldface**). The tree was constructed from partial nucleotide sequences of the capsid region by using PEC strain (a porcine SaV) as an outgroup. The numbers on each branch indicate the bootstrap values. Scale bar indicates nucleotide substitutions per position. GenBank accession numbers of reference strains are as follows: Osaka07-767/08/JP (AB433785), Osaka/19-115/07/JP (AB327280), Sydney3/04/AU (DQ104357), CU050202/05/HK (DQ155647), Angelholm/SW278/04/SE (DQ125333), Chiba/000671/99/JP (AJ786349), 18/DCC/Tokyo/43/JP (AB236378), Yak2/00/JP (AB046353), Ehime1596/99/JP (AM049952), CU050107/ 05/HK (DQ155646), CMH044/03/03/THA (EF600796), St. Petersburg/8214/05/RUS (FJ214057), Karachi/730/92/PK (AB126249), Hou7-1181/90/USA (AF435814), Osaka/5836/04/JP(AB242324), Karachi/872/91/PK (AB181231), Sapporo/82/JP (U65427), Manchester/ 93/UK (X86560), 12/DCC/Tokyo/44/JP (AB235380), 4724/Osaka/02/JP (AB180212), Stockholm/318/97/SE (AF194182), Mex14917/00/USA (AF435813), Karachi/867/93/PK (AB181132), Parkville/94/UK (U73124), Houston/90/USA (U95644), Karachi/1017/90/PK (AB181227), Arg39/95/ARG (AY289803), Lyon/598/97/F (AJ271056), London/29845/92/UK (U95645), Mex340/90/USA (AF435812), Cruise ship/00/USA (AY289804), Karachi/874/92/PK (AB181129), Karachi/928/94/PK (AB181128), 4408/Maizuru/03/JP (AB180209), and PEC (AF182760).

Conclusions

According to the past 5 years of SaV surveillance conducted in the same setting and population in Japan, SaV GI/1 was the most common genotype during 2003–2004, and thereafter genotype GI/6 dominated over the GI/1 in 2004–2005 (*2*). Then, the GI/6 genotype was replaced by the predominant SaV GI/1 since 2005 until 2007 (*7*; S.K. Dey, unpub. data). Although GIV SaV strains had been isolated in some countries, including Japan, Thailand, Pakistan, and Hong Kong Special Administrative Region of the People's Republic of China (*4,8–11*), they were detected less often than strains from the other genogroups, and whether they are the recombinant strains has not been confirmed. These findings demonstrate the changing epidemiology of SaV genogroup with the emergence of intergenogroup recombinant SaVs (GII/GIV) and the sudden decrease of predominant SaV GI in Japan during 2007–2008.

Recently, several types of recombinant SaV strains, including intergenogroup and intragenogroup, have occurred frequently in Japan (*5,12–14*), which indicates that recombination between SaV genomes is another important feature of the evolution of SaV. Although factors contributing to the emergence of these recombinant stains during the period of surveillance are not known, the recombinant strains possibly appeared when the pediatric population might have lacked antibody protection to these strains.

Our results suggest that recombinant strains may be underestimated. Characterization of SaVs usually is based on the capsid gene sequence only, whereas both the capsid gene and RNA polymerase sequences are needed to identify such viruses. Furthermore, constant surveillance is important to successfully monitor the emergence of these strains.

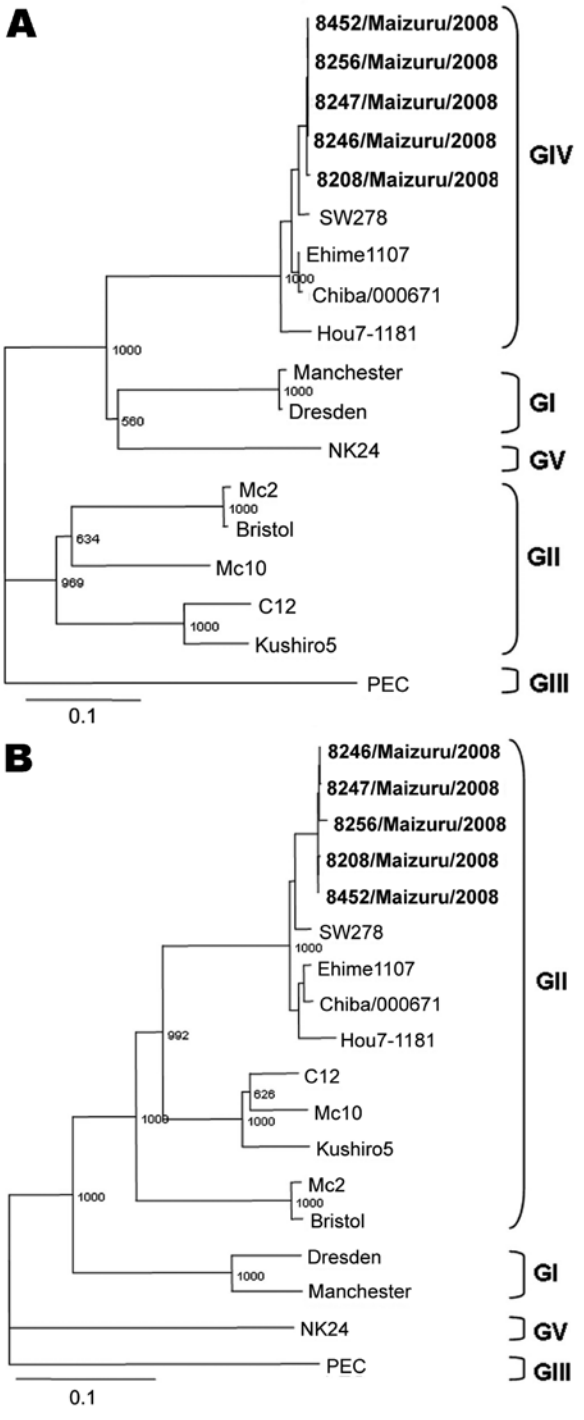

Figure 2. Phylogenetic analysis of the polymerase region (A) and the capsid region (B), showing the different genogroups (GI–GV). The sapovirus (SaV) isolates detected in the study are highlighted in **boldface**. The scale indicates nucleotide substitutions per position. The numbers in the branches indicate the bootstrap values. GenBank accession numbers of reference strains are as follows: C12 (AY603425), Mc10 (AY237420), Kushiro5 (AB455793), Mc2 (AY237419), Bristol (AJ249939), Dresden (AY694184), NK24 (AY646856), Ehime1107 (DQ058829), Yak2 (AB046353), and Yokohama/16/2007/JP (AB305049).

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Lack of Macrolide Resistance in *Chlamydia trachomatis* after Mass Azithromycin Distributions for Trachoma

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We investigated antimicrobial drug resistance in ocular *Chlamydia trachomatis* 18 months after 4 biannual communitywide distributions of antimicrobial drugs in a region of Ethiopia where ocular strains of *C. trachomatis* are highly endemic. We found no significant differences in susceptibilities to azithromycin and doxycycline in 6 posttreatment and 4 pretreatment samples.

Trachoma, a sequela of repeated conjunctival infection with *Chlamydia trachomatis*, is the leading cause of infectious blindness (*1*). To control endemic trachoma, the World Health Organization (WHO) recommends communitywide distribution of antimicrobial agents, along with surgery and improved hygiene. Mass azithromycin treatments have been effective in reducing this infection (*2*). However, concerns have been raised that selective antimicrobial pressure may produce macrolide-resistant strains of *C. trachomatis* and other pathogens (*3*).

Solomon et al. reported on antimicrobial drug susceptibility in 9 chlamydial isolates 2 months after mass antimicrobial drug treatment in Tanzania (*4*). These authors observed a slight increase in the median MIC after treatment but found no resistant strains. Despite this encouraging study, investigations of the long-term impact of multiple treatments on antimicrobial drug susceptibility are needed. Geographic areas where trachoma is hyperendemic require repeated mass distributions because infection has been shown to return after a single treatment (*5*). Antimicrobial drug–resistant *C. trachomatis* might not emerge until multiple treatments have occurred. For example, in Nepal, azithromycin-resistant pneumococcal strains were observed only after consecutive annual treatments (*3*). The selective pressure of repeated azithromycin distributions might enable rapid expansion of resistant clones (*6*). In 2006, we investigated antimicrobial drug resistance in ocular *C. trachomatis* 18 months after 4 biannual treatments (2003–2004) in a trachoma-endemic region of Ethiopia.

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The Study

We obtained ethical approval from the Committee on Human Research at the University of California, San Francisco (UCSF), and from the National Ethical Clearance Committee of the Ethiopian Science and Technology Agency. Antimicrobial treatments were distributed every 6 months to 24 randomly selected villages in the Gurage zone in Ethiopia. Persons ≥1 year of age were offered single-dose oral azithromycin (1 g for adults or 20 mg/kg for children) as directly observed treatment. Pregnant women and those allergic to macrolides were offered a 6-week course of topical 1% tetracycline ointment (applied 2×/day to both eyes, not directly observed). At each biannual visit, 8 villages not yet receiving antimicrobial distribution were randomly selected from the same district to serve as controls (*5*). The same procedures were conducted in the control villages as in the treatment villages. After completion of clinical ocular examinations and sample collection, treatment was offered to study and control villages.

All children 1–5 years of age received ocular examinations, and conjunctival swabs were taken from their right tarsal conjunctiva before treatment. Swabs were placed immediately in M4RT media (Remel, Lenexa, KS, USA) at 4°C, frozen at –20°C within 6 hours, and transported at 4°C to the microbiology laboratory at the Proctor Foundation at UCSF, where they were stored at –80°C until PCR was performed. An aliquot of each sample was processed with the Amplicor PCR test (Roche Molecular Systems, Branchburg, NJ, USA) for detection of *C. trachomatis*. At 18 months after the fourth biannual treatment, the prevalence of ocular infection in preschool children was 10.7% (95% confidence interval [CI] 5.5%–19.3%) (*7*). In control villages that had not yet received treatment, the average prevalence was 31.2% (95% CI 23.1%–40.5%). Of 552 samples from 8 biannually treated villages, 59 were positive by PCR. Of 523 samples from 8 control villages, 163 were PCR positive. Among these PCR-positive samples, 10 were randomly chosen from biannually treated villages and 10 from control villages. Remnants of the original specimens were sent to the UCSF Chlamydia Laboratory for culture and antimicrobial susceptibility testing.

Technicians were unaware of the origin of each sample, and the order in which samples were tested was randomized. *C. trachomatis* isolation was carried out in cycloheximide-treated McCoy cells in 1-dram shell vials by using a modification of the procedure of Ripa and Mardh (*8*). We sequenced the *ompA* gene from all conjunctival isolates (*9*).

The protocol for determining antimicrobial drug sensitivity (*10*) was used with minor modifications. Azithromycin (American Pharmaceutical Partners, Schaumburg, IL, USA) and doxycycline (Bedford Laboratories, Bedford, OH, USA) were tested against chlamydia isolates; MICs were conducted in triplicate, and minimum chlamydicidal concentrations (MCCs) in duplicate. Each MIC or MCC included a clinical chlamydia isolate with a known susceptibility to azithromycin and doxycycline as a positive control to ensure the validity of the sensitivity testing.

Among 10 Amplicor PCR–positive samples randomly chosen from biannually treated villages, cultures for 7 samples were positive; 6 of these were tested for resistance against azithromycin and doxycycline (Table). Of 10 random Amplicor PCR–positive samples from pretreatment villages, cultures for 6 were positive; 4 of these samples were tested for resistance (Table).

MICs and MCCs were comparable between biannually treated villages and control villages for the 2 antimicrobial drugs tested. No statistically significant differences were found ($p = 0.76$ for azithromycin MIC; $p = 1.00$ for azithromycin MCC; $p = 0.22$ for doxycycline MIC; and $p = 0.45$ for doxycycline MCC) (Wilcoxon rank-sum test, STATA, StataCorp, College Station, TX, USA). With the number of samples tested (6 posttreatment samples and 4 pretreatment samples), we had 95% power to detect a 2-fold shift in the mean MIC ($\alpha = 0.05$, two-sided) and 90% power to detect a 2-fold shift in the mean MCC of azithromycin. For doxycycline, we had 99% power to detect a 2-fold change in the mean MIC ($\alpha = 0.05$, two-sided) and 85% power to detect a 2-fold change in the mean MCC.

Table. Antimicrobial drug susceptibilities of *Chlamydia trachomatis*, Ethiopia*

Treatment status	Serotype	Azithromycin†		Doxycycline†	
		MIC	MCC	MIC	MCC
4 biannual	A/Har13	0.5	0.5	0.03	0.03
	Ba/Apache-2	0.5	0.5	0.03	0.03
	Ba/Apache-2	0.5	0.5	0.03	0.06
	Ba/Apache-2	0.25	0.5	0.03	0.06
	Ba/Apache-2	0.5	0.5	0.03	0.06
	Ba/Apache-2	0.5	0.5	0.03	0.03
None	A/Har 13	0.5	0.5	0.03	0.03
	A/Har 13	0.5	0.5	0.03	0.03
	A/Har 13	0.5	1	0.015	0.06
	Ba/Apache-2	0.25	0.25	0.03	0.03

*Samples taken in 2006, 18 months after 4 biannual treatments in 2003–2004. MCC, minimum chlamydicidal concentration.
†MIC and MCC values given in μg/mL.

Conclusions

This study found no significant increase in antimicrobial drug resistance against azithromycin or doxycycline after treatment for ocular *C. trachomatis*. Our study had limited power to detect a rare mutation that can engender resistant chlamydia strains but sufficient power to detect a major shift in antimicrobial susceptibilities. In a region like Ethiopia, where trachoma is endemic, infection often returns to the community after mass distribution of antimicrobial drugs stops (*7*). Concerns exist that resistant strains may result in failure to eliminate trachoma locally, but our study refutes this concern. The lack of antimicrobial drug resistance shown in this study is particularly encouraging because the region was subjected to higher selective pressures from biannual treatments than would be expected from annual treatments recommended by WHO.

Chlamydiae are obligate intracellular bacteria that can multiply only in the cytoplasm of a susceptible host cell, and acquisition of antimicrobial resistance genes from other organisms through horizontal transfer is probably rare. In vitro spontaneous mutations in chlamydia can confer antimicrobial resistance, including that in the 23S rRNA, but mutations appear to incur competitive disadvantages (*11*).

The persistent pressure of repeated mass treatments may select for compensatory mutations that could adapt chlamydia to the fitness cost of the initial mutation (*11*). This scenario would enable the expansion of resistant strains after communitywide distribution of azithromycin in trachoma control programs. However, after treatment is discontinued, sensitive wild type strains may outcompete resistant ones. In one Australian community, the increased population of azithromycin-resistant pneumococcus was quickly replaced by sensitive strains after treatment ceased (*6*). In our study, if resistant strains emerged after 4 biannual antimicrobial treatments, they did not survive the competition with sensitive strains 18 months after mass treatment stopped.

Determining antimicrobial resistance in *C. trachomatis* is technically challenging, has no standardized protocol (*12*), and is rarely pursued. DNA sequencing to identify genetic mutations that confer macrolide resistance could be more sensitive but would require further efforts to characterize specific mutations causing macrolide resistance. Mass distribution of antimicrobial drugs, especially azithromycin, is a critical component in the global elimination of blinding trachoma. Emergence of antimicrobial drug resistance to chlamydia treatments would be detrimental to the goal of eliminating trachoma, so surveillance for resistance should be part of trachoma programs.

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Genetically Diverse Coronaviruses in Wild Bird Populations of Northern England

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Infectious bronchitis virus (IBV) causes a costly respiratory viral disease of chickens. The role of wild birds in the epidemiology of IBV is poorly understood. We detected diverse coronaviruses by PCR in wildfowl and wading birds in England. Sequence analysis showed some viruses to be related to IBV.

Infectious bronchitis virus (IBV), a group 3 coronavirus, causes a costly viral disease of chickens that is found worldwide (*1*). It can cause respiratory disease in chickens of all ages and a loss of production and egg quality in mature hens (*2*). Some strains are nephropathogenic, resulting in renal-induced mortality rates of up to 25% for susceptible flocks (*1*). Currently, the poultry industry controls disease through the use of vaccines. However, IBV continuously generates antigenic variants; current vaccines offer no protection against some of them (*3*). Wild birds may play a role as both reservoirs and long-distance vectors of IBV.

Recently, group 3 coronaviruses genetically similar to IBV were detected in healthy galliform and nongalliform birds (*4*,*5*). This finding may suggest that wild birds are able to carry IBV-like viruses asymptomatically. Other studies have detected coronaviruses that are genetically distinct from IBV in wild birds, including graylag geese (*Anser anser*), rock doves (*Columba livia*), mallards (*Anas platyrhynchos*), Chinese bulbuls (*Pycnonotus sinensis*), red-whiskered bulbuls (*Pycnonotus jocosus*), gray-backed thrushes (*Turdus hortulorum*), blackbirds (*Turdus merula*), white-rumped munias (*Lonchura striata*), and scaly-breasted munias (*Lonchura punctulata*) (*6*,*7*). We report the detection and characterization of group 3 coronaviruses, some of which appear to be related to IBV, from wild bird populations sampled in northern England.

The Study

Serial cross-sectional surveys of wild bird populations throughout northern England were undertaken from July 2004 through January 2007. Samples were collected from 441 individual wild birds of 42 species, including both migratory and resident species (Table 1). Fecal samples and oropharyngeal swabs were collected from live birds that had been caught primarily for ringing purposes (*8*) and from dead wildfowl and corvids provided by licensed shooters. Biometric data and information concerning the location where birds had been trapped or shot were recorded (*8*). Samples were placed in virus transport media (Eagle minimum essential medium supplemented with 10% fetal calf serum, penicillin, and streptomycin [10,000 units penicillin, 10 μg streptomycin/mL] and amphotericin B [250 μg/mL]); samples collected from the same wild bird species or genus were pooled in groups of 5 and frozen at –80°C until required.

Viral RNA was extracted from pooled fecal samples and pooled oropharyngeal swabs by using a QIAamp Viral RNA Mini Kit (QIAGEN, Crawley, UK) following the manufacturer's instructions. Reverse transcription–PCR was used to detect avian coronaviruses as previously described (*9*). The primers UTR41+ (5′-ATGTCTATCGCCAGGGAAATGTC-3′) and UTR11- (5′-GCTCTAACTCTATACTAGCCTA-3′) targeted the 3′ untranslated region (UTR) of the coronavirus genome, which is highly conserved among all known types of IBV (*9*). This procedure was followed by use of a heminested PCR with the same forward primer but the reverse primer UTR hemi- (5′-CTTAAACTAAAATTTAGCTCTTCC-3′) under the same reaction conditions as the initial PCR, which had an expected product size of 214 bp. PCR products were purified by using a commercial purification kit (QIAquick PCR Purification Kit; QIAGEN) according to the manufacturer's instructions and were sequenced commercially (Cogenics, Essex, UK) as recommended by the manufacturer (ABI 3730xl DNA Analyser; Applied Biosystems, Warrington, UK). Nucleotide sequences derived from this study have been deposited in the GenBank sequence database under accession nos. FJ490193–FJ490199.

Sequences were aligned with previously published coronavirus sequences obtained from GenBank by using the Clustal program within the MEGA 4.0 package (*10*) (online Appendix Figure, available from www.cdc.gov/EID/content/15/7/1091-appF.htm). Phylogenetic analyses were conducted in MEGA 4.0. Phylogenies were estimated by using a minimum-evolution method (*11*), and evolutionary distances were computed by using the Tamura-Nei method (*12*). Phylogenetic trees were drawn to scale; branch lengths in the same units as those of the evolutionary distances were used to infer the phylogenetic tree. Bootstrap analysis using 1,000 repetitions provided support for individual nodes (*13*).

Animal-level prevalences and confidence limits, based on pooled samples, were estimated by using a pooled prev-

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Table 1. Wild bird species from which oropharyngeal swabs and fecal samples were collected and screened for coronavirus RNA, England

Taxonomic family	Common name	Latin name	No. birds screened
Sulidae	Northern gannet	*Morus bassanus*	1
Phalacrocoracidae	Great cormorant	*Phalacrocorax carbo*	2
Ardeidae	Grey heron	*Ardea cinerea*	35
Anatidae	Whooper swan	*Cygnus cygnus*	55
	Mute swan	*Cygnus olor*	25
	Pink-footed goose	*Anser brachyrhynchus*	3
	Greylag goose	*Anser anser*	1
	Canada goose	*Branta canadensis*	1
	Brent goose	*Branta bernicla*	7
	Common shelduck	*Tadorna tadorna*	2
	Mallard	*Anas platyrhynchos*	34
	Northern pintail	*Anas acuta*	9
	Northern shoveler	*Anas clypeata*	1
	Eurasian wigeon	*Anas penelope*	33
	Common teal	*Anas crecca*	18
	Common pochard	*Aythya ferina*	10
Accipitridae	Eurasian sparrowhawk	*Accipiter nisus*	4
Falconidae	Common kestrel	*Falco tinnunculus*	1
Rallidae	Common moorhen	*Gallinula chloropus*	1
	Common coot	*Fulica atra*	13
Haematopodidae	Eurasian oystercatcher	*Haematopus ostralegus*	42
Charadriidae	Ringed plover	*Charadrius hiaticula*	5
Scolopacidae	Dunlin	*Calidris alpina*	8
	Sanderling	*Calidris alba*	8
	Red knot	*Calidris canutus*	14
	Ruddy turnstone	*Arenaria interpres*	26
	Common redshank	*Tringa totanus*	3
	Eurasian woodcock	*Scolopax rusticola*	3
Laridae	Black-headed gull	*Larus ridibundus*	7
	Herring gull	*Larus argentatus*	15
	Great black-backed gull	*Larus marinus*	1
	Lesser black-backed gull	*Larus fuscus*	2
Sternidae	Common tern	*Sterna hirundo*	25
Alcidae	Common guillemot	*Uria aalge*	1
Columbidae	Rock dove	*Columba livia*	2
	Eurasian collared dove	*Streptopelia decaocto*	5
Tytonidae	Barn owl	*Tyto alba*	4
Prunellidae	Dunnock/Hedge accentor	*Prunellla modularis*	1
Turdidae	Northern wheatear	*Oenanthe oenanthe*	2
Passeridae	House sparrow	*Passer domesticus*	4
Fringillidae	European greenfinch	*Carduelis chloris*	2
	Eurasian siskin	*Carduelis spinus*	5

alence calculator (www.ausvet.com.au/pprev). Generalized linear modeling was used to calculate maximum-likelihood estimates of prevalence and confidence limits (*14*).

Coronavirus RNA was detected in 7 fecal sample pools (Table 2), giving an individual animal–level prevalence estimate of 1.6% (95% confidence interval 0.7–3.1). Of the pools with positive results for coronavirus, 4 were collected from ducks (designated Anas/UK/p20/2005, Anas/UK/p33/2005, Anas/UK/p42/2005 and Anas/UK/p71/2005; Table 2). Another pool contained samples from whooper swans (*Cygnus cygnus*) (whooper swan/UK/p3/2005), 1 consisted of samples from red knots (*Calidris canutus*) (red knot/UK/p60/2006), and 1 contained samples from Eurasian oystercatchers (*Haematopus ostralegus*) (oystercatcher/UK/p17/2006). PCR-positive pools were from birds sampled in estuarine, salt marsh, or standing water habitats (Table 2). All birds appeared to be healthy. All pooled oropharyngeal samples were PCR negative.

Phylogenetic analyses were based on a final usable sequence of 146 nt after removal of primer sites (online Appendix Figure). Nucleotide distances between coronavirus sequences derived from this study were 0.0%–15.6%. Sequences detected in 3 pooled duck samples and a sequence derived from a pool of whooper swan samples clustered

Table 2. Characteristics of wild birds that contributed to CoV RT-PCR–positive fecal pools*

Pool	Source species	Habitat†	Age	Sex	CoV detected
1	Whooper swan	Standing fresh water	Juvenile	M	Whooper swan/UK/p3/2005
	Whooper swan	Standing fresh water	Adult	M	
	Whooper swan	Standing fresh water	Adult	M	
	Whooper swan	Standing fresh water	Adult	M	
	Whooper swan	Standing fresh water	Adult	F	
2	Teal	Estuarine salt marsh	Juvenile	F	Anas/UK/p20/2005
	Mallard	Estuarine salt marsh	Unknown	M	
	Mallard	Estuarine salt marsh	Unknown	M	
	Mallard	Estuarine salt marsh	Unknown	M	
	Wigeon	Estuarine salt marsh	Juvenile	F	
3	Pintail	Estuarine salt marsh	Unknown	F	Anas/UK/p33/2005
	Pintail	Estuarine salt marsh	Unknown	M	
	Wigeon	Estuarine salt marsh	Unknown	F	
	Mallard	Estuarine salt marsh	Unknown	F	
	Mallard	Estuarine salt marsh	Unknown	F	
4	Pintail	Estuarine salt marsh	Unknown	F	Anas/UK/p71/2005
	Wigeon	Estuarine salt marsh	Unknown	M	
	Pintail	Estuarine salt marsh	Unknown	M	
	Wigeon	Estuarine salt marsh	Unknown	M	
	Wigeon	Estuarine salt marsh	Unknown	M	
5	Red knot	Estuarine	Unknown	Unknown	Red knot/UK/p60/2006
	Red knot	Estuarine	Unknown	Unknown	
	Red knot	Estuarine	Unknown	Unknown	
	Red knot	Estuarine	Unknown	Unknown	
	Red knot	Estuarine	Unknown	Unknown	
6	Oystercatcher	Estuarine	Adult	Unknown	Oystercatcher/UK/p17/2006
	Oystercatcher	Estuarine	Juvenile	Unknown	
	Oystercatcher	Estuarine	Adult	Unknown	
	Oystercatcher	Estuarine	Adult	Unknown	
	Oystercatcher	Estuarine	Adult	Unknown	
7	Wigeon	Estuarine salt marsh	Unknown	F	Anas/UK/p42/2005
	Wigeon	Estuarine salt marsh	Unknown	F	
	Wigeon	Estuarine salt marsh	Unknown	F	
	Wigeon	Estuarine salt marsh	Unknown	F	
	Wigeon	Estuarine salt marsh	Unknown	M	

*CoV, coronavirus; RT-PCR, reverse transcription–PCR.
†Habitat type in which wild bird was caught for sampling.

with sequence from an IBV H120 (Massachusetts) vaccine strain. Sequences within this cluster were relatively homogenous with low within-group distance values (0.0%–2.8%). Within this cluster, bootstrap support for the individual nodes was relatively low (Figure).

Coronavirus sequences detected in red knots clustered with a previously described goose coronavirus; divergence at the nucleotide level was 2.0%. Sequences from viruses detected in samples from Eurasian oystercatchers and ducks clustered with sequence from a published duck coronavirus (*7*). The sequence from Eurasian oystercatchers was identical to that of the published duck coronavirus; distance values within this cluster were 0.0%–1.0%.

Conclusions

Although samples were collected from wild bird populations comprising 46 species of wild birds from numerous and diverse habitats, coronavirus RNA was detected only in wildfowl (Anseriforms) and waders (Charadriiformes). Coronaviruses have been detected previously in wildfowl species, rock doves, wild peafowl, and some passerine species (*4*–*7*). All wild birds from which coronaviruses were detected in this study appeared to be healthy. Although IBV is recognized primarily as a respiratory agent, it has been demonstrated that certain strains are able to replicate in the chicken intestine without obvious clinical disease (*15*).

Phylogenetic analysis showed that coronavirus sequences detected by this study were genetically diverse. Virus sequences from 3 pools of fecal samples from ducks and whooper swans shared high nucleotide sequence identity with sequence from the IBV H120 vaccine strain, which is commonly used for the vaccination of commercial chickens worldwide. Coronaviruses sharing a high degree of identity with the IBV H120 vaccine strain have been detected previously in healthy, unvaccinated, domestic peafowl and as well as wild peafowl in China (*4*,*5*). These vi-

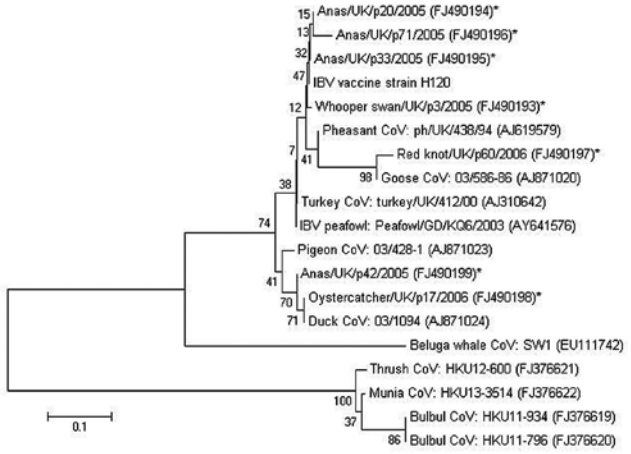

Figure. Minimum-evolution tree (*11*) of coronaviruses based on a 146-bp fragment of the 3′ untranslated region of infectious bronchitis virus (IBV). Evolutionary distances were computed by using the Tamura-Nei method (*12*) and are in the units of the number of base substitutions per site. Coronaviruses detected in wild birds by this study are denoted with an asterisk. Previously published coronavirus sequences from different sources were included for comparative purposes. GenBank accession numbers are shown in brackets. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (*13*). The tree is drawn to scale; branch lengths are in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic analyses were conducted in MEGA4 (*10*). CoV, coronavirus. Scale bar indicates nucleotide substitutions per site.

ruses may be revertant attenuated vaccine strains that have arisen as a result of the widespread use of IBV vaccines in the local poultry population in China. To understand their potential role as reservoirs of IBV strains, further surveillance for coronaviruses in wild bird populations is needed. It would be useful to determine the number and genome position of accessory genes of the coronaviruses detected in wild birds and to compare them with those of IBV. More detailed genetic characterization of the viruses detected including, for example, the S1 spike gene, is also needed. The detection of coronaviruses that appear to be related to IBV in wild migratory birds raises interesting questions as to their role in the transmission, dissemination, and evolution of IBV strains.

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WU Polyomavirus in Patients Infected with HIV or Hepatitis C Virus, Connecticut, USA, 2007

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WU polyomavirus (WUPyV) was detected in 10 (8.3%) of 121 HIV-positive plasma specimens, 0 (0%) of 120 HIV-negative serum specimens, and 2 (2.5%) of 79 hepatitis C virus (HCV)–positive serum specimens. KI polyomavirus was not detected in HIV-positive plasma or HCV-positive serum specimens. HIV-infected persons may be susceptible to systemic WUPyV infection.

In 2007, 2 new human polyomaviruses, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), were identified. KIPyV was initially detected in an extract obtained from 20 pooled randomly selected nasopharyngeal aspirates, and WUPyV was detected in a nasopharyngeal aspirate from a 3-year-old child from Australia who had a diagnosis of pneumonia (*1,2*). These viruses have since been detected in respiratory tract specimens from symptomatic and asymptomatic children, although no clear association with respiratory disease has been demonstrated (*3–6*).

Previously identified human polyomaviruses (BK virus [BKV] and JC virus [JCV]) cause clinical disease in immunocompromised persons (*7,8*). Although viremia may be associated with immunosuppression, correlation of JCV DNA in peripheral blood with development of progressive multifocal encephalopathy in AIDS patients remains controversial (*9*). BKV DNA has been detected in blood of renal transplant patients, and BKV load may be predictive of polyomavirus-associated nephropathy (*10*).

The Study

The pathogenesis and clinical spectra of WUPyV and KIPyV, particularly in immunocompromised persons, have not been defined. To investigate whether WUPyV or KIPyV is present in persons with chronic viral infection and perhaps compromised immunity, we conducted a cross-sectional study in which we screened the following for WUPyV and KIPYV DNA: plasma samples from HIV-infected persons, serum samples from hepatitis C virus (HCV)–infected persons, and a control group of HIV-negative persons.

Three groups of samples submitted to the Clinical Virology Laboratory at Yale–New Haven Hospital in 2007 were screened: HIV PCR-positive plasma, HCV PCR-positive serum, and HIV antibody-negative serum. Patient identifiers were removed and these specimens were tested as part of our ongoing investigation for newly identified viruses. Collection of specimens and clinical data was approved by the Yale University Human Investigation Committee and was compliant with Health Insurance Portability and Accountability Act regulations.

Nucleic acids were extracted from each specimen by using QIAamp nucleic acid purification kits (QIAGEN, Valencia, CA, USA). Screening for WUPyV DNA has been described (*6*). Briefly, we performed an initial PCR screening specific for the virus capsid protein 2 (VP2) gene by using primers described by Gaynor et al. (*2*) and a nested PCR (Table 1, primers 1 and 2). To confirm results, DNA from all PCR-positive samples was reextracted and screened with primers specific for the region of the genome containing the noncoding control region (NCCR), which includes the virus origin of replication (genome coordinates 5213 to nt 36 of the circular viral genome). This screening included an initial PCR (Table 1, primers 3 and 4) and a nested PCR (Table 1, primers 5 and 6). The nested PCR generated a 328-bp amplicon. Screening for KIPyV DNA by PCR included a nested PCR specific for the VP1 gene according to the protocol described by Allander et al. (*1*). Positive and negative controls were included in each set of PCRs.

All PCR products were sequenced by using 377 DNA automated sequencers (Applied Biosystems, Foster City, CA, USA) at the W.M. Keck Biotechnology Resource Laboratory at Yale University School of Medicine. For WUPyV, phylogenetic analysis was performed on a 194-bp fragment within the amplified region of NCCR (nt 5197 to nt 159 of the circular viral genome) by using Lasergene MegAlign software (DNASTAR Inc., Madison, WI, USA) (ClustalW alignment method). The only clinical data available for these deidentified serum specimens were HIV/HCV status and virus loads. HIV and HCV virus loads were determined in the Clinical Virology Laboratory by quantitative reverse transcription–PCR using commercially available diagnostic tests. The Fisher exact test was used to determine whether the difference in the percentage of WUPyV-positive specimens in HIV-positive and HIV-negative patients was statistically significant.

Ten (8.3%) of 121 HIV-positive specimens and 0 (0%) of 120 HIV-negative samples were positive for WUPyV ($p<0.01$) (Table 2). Two (2.5%) of 79 HCV-positive samples were positive for WUPyV. One of the 10 WUPyV-

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Table 1. Primers used in PCR to detect WU polyomavirus in serum specimens, Connecticut, USA, 2007

Primer	Name	Sequence (5′ → 3′)	Genome coordinates
1	WU2F*	GCGCATCAAGAGGCACAGCTACTATTTC	1377–1400
2	WU2R*	GCGCCTAGCCTGTGAACTCCATC	1510–1528
3	WUoriFnest†	CTCATTTCCCCCTTTGTCAGGATG	5011–5034
4	WUoriRII†	CTTTCCGCTGGACTACAAAGGGC	317–339
5	WUoriF†	GTAAATTTCCCCAGCAGGTC	5075–5095
6	WUoriR†	CGGAAACTTTAAAAGGTCACAG	153–174

*Primers used by Wattier et al. (*6*).
†The amplicon generated with these primer spans across nt 1 of the 5,229-bp circular virus genome.

positive, HIV-positive specimens was also positive for HCV. Current HIV status for 2 of the WUPyV-positive, HCV-positive persons was not available. None of the HIV-positive or HCV-positive specimens screened were positive for KIPyV (Table 2). HIV-negative specimens were not screened for KIPyV.

Mean HIV loads for WUPyV-positive and WUPyV-negative, HIV-positive persons were 32,200 copies/mL (range 2,930–88,300 copies/mL, median 25,100 copies/mL) and 131,500 copies/mL (range 509–750,000 copies/mL, median 59,600 copies/mL), respectively. Mean HCV loads for WUV-positive and WUV-negative persons were 1,302,200 copies/mL (range 88,600–3,906,600 copies/mL, median 1,909,000 copies/mL) and 3,091,000 copies/mL (range 2,030–32,800,000 copies/mL, median 1,150,000 copies/mL), respectively.

Amplified sequences from WUPyV-positive serum specimens were compared with available sequences from GenBank (all of which were obtained from respiratory specimens). Several nucleotide polymorphisms were observed in the amplified portion of the NCCR of the New Haven WUPyV serum isolates (Figure). None of the nucleotide changes in the New Haven strains were mapped to the 4 putative large T-antigen binding sites within the origin of replication (*2*).

Conclusions

We detected WUPyV in plasma specimens from HIV-infected persons and in serum specimens from HCV-infected persons in Connecticut in 2007. Detection of WUPyV indicates that this newly identified polyomavirus is present in peripheral blood of HIV-positive or HCV-positive persons. It does not appear that WUPyV viremia correlates with HIV or HCV virus load. We did not assess the WUPyV serologic status of these persons. However, if the seroepidemiology of WUPyV is similar to that of BKV and JCV, we predict that >85% of the persons screened will have detectable antibodies to WUPyV (*11*). Whether antibody status for WUPyV correlates with viremia is unknown. Viremia may represent primary infection or reactivation of latent infection.

Viremia has been described for JCV and BKV. JCV DNA in serum/plasma may correlate with the degree of immunosuppression. However, blood from viremic persons infected with JCV has a low positive predictive value for development of progressive multifocal encephalopathy in AIDS patients (*9*). A recent study suggested that screening for BKV replication is useful in identifying patients at risk for BKV-associated nephropathy, which may enable early interventions such as renal biopsy and reduction of immunosuppression (*12*). However, because of the lack of clinical data available for WUPyV-positive persons in our study, it was not possible to make any clinical correlations.

The absence of KIPyV in HIV-positive or HCV-positive peripheral blood specimens suggests that host susceptibility for KIPyV may differ from that of WUPyV, as for JCV and BKV. However, this hypothesis was not supported by a recent study that reported KIPyV and WUPyV in autopsy lymphoid tissues of AIDS patients (*13*). Whether WUPyV or KIPyV cause disease in HIV-positive persons or other populations remains to be determined.

Our data demonstrate that WUPyV was detected in peripheral blood of HIV- and HCV-infected persons. However, the scope of this study was limited because clinical data were not available for study participants. Whether WUPyV or KIPyV have oncogenicity or other pathogenicity in immunocompromised hosts remain to be determined. The role of polyomaviruses in human cancers has been extensively investigated but conclusive evidence is lacking (*14*). The genome of Merkel cell polyomavirus, a new polyomavirus, was found to be integrated within the cellular genome of Merkel cell carcinoma tissue samples, which suggests a role for this virus in a specific tumor (*15*). Therefore, studies to assess the oncogenic potential of WUPyV and KIPyV are also needed.

Table 2. Detection of WU and KI polyomaviruses in HIV-positive plasma, HIV-negative serum, and HCV-positive serum specimens, Connecticut, USA, 2007*

Specimen group	No. WU virus–positive specimens/total no. specimens tested (%)	No. KI virus–positive specimens/total no. specimens tested (%)
HIV+	10/121 (8.3)†	0/120
HIV–	0/120 (0)	Not tested
HCV+	2/79 (2.5)	0/80

*HCV, hepatitis C virus.
†p<0.01 vs. HIV– samples.

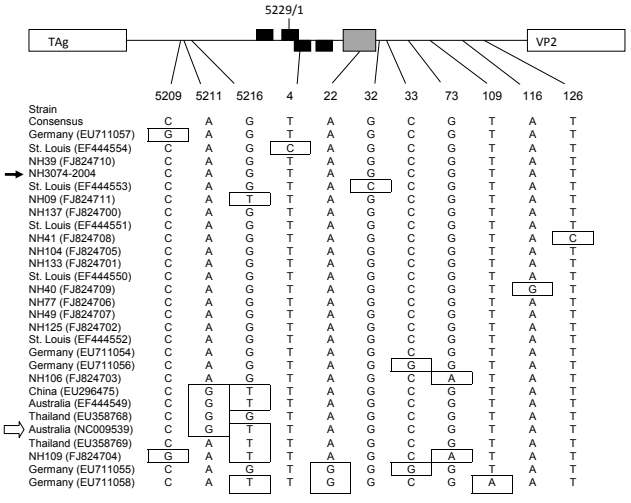

Figure. Nucleotide polymorphisms in the noncoding region of WU polyomavirus (WUPyV), Connecticut, USA, 2007. Sequences spanning nt 5197 to 159 of the circular viral genome of New Haven human serum isolates and all available sequences from GenBank (all from respiratory specimens) were subjected to phylogenetic analysis. A map of the noncoding region within the viral genome is indicated at the top of the figure (not to scale). The arbitrary last (5229) and first (1) nucleotide of the circular viral genome are indicated. The putative large T antigen (TAg) binding sites for each strand of the double-strand genome are indicated by black boxes, and the A/T-rich region is indicated by a gray box. Position of nucleotide polymorphism is indicated below the map. Strains (location and GenBank accession nos.) are listed in order according to phylogenic analysis. The consensus sequence is listed first, and polymorphisms are indicated by boxes. The strain indicated by the black arrow is a WUPyV New Haven respiratory isolate (*6*), and the strain indicated by the white arrow is the original WUPyV isolate (*2*). VP2, virus capsid protein 2.

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Methicillin-Resistant *Staphylococcus aureus* ST398 in Swine Farm Personnel, Belgium

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We assessed methicillin-resistant *Staphylococcus aureus* (MRSA) in persons on 49 swine farms in Belgium. Surveys showed that 48 (37.8%) persons carried MRSA ST398 and 1 (0.8%) had concurrent skin infection. Risk factors for carriage were MRSA carriage by pigs, regular contact with pigs and companion animals, and use of protective clothing.

Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) carriage has been high (>10%) among swine and exposed farmers and veterinarians (*1*,*2*). These MRSA strains are genetically unrelated to hospital- or community-acquired clones. They are resistant to digestion with *Sma*I and belong to ST398 (*1*). We assessed prevalence and characteristics of MRSA carriage and infection and associated risk factors for swine farm residents and workers in Belgium during 2007.

The Study

We randomly selected 50 farms from the 7,500 farms in the pig farm national database of the Belgian Federal Agency for Food Safety. Sample size was based on an estimated 20% prevalence of MRSA colonization among farm workers and residents (*1*). The sample size was calculated to test the null hypothesis of prevalence <10% with a power of 80%. Participation was offered to all persons on the farm at the time of the visit, including farmers, co-workers, and household members. After giving written consent, participants were screened for MRSA carriage and interviewed. Simultaneously, on the same farms, a study of MRSA carriage in 30 randomly selected pigs per farm was conducted by veterinarians (*3*).

Samples from anterior nares and skin lesions on hands or face of human participants were placed into Stuart transport medium (Copan, Italy), inoculated within 24 h into 7.5% NaCl brain–heart infusion enrichment broth, and subcultured after 24 h onto Chromagar MRSA (bioMérieux, Marcy l'Etoile, France) and mannitol salt agar (Becton Dickinson, Heidelberg, Germany). *S. aureus* isolates were identified by coagulase test and PCR for 16S rRNA, *mecA,* and *nuc* genes (*4*).

Isolates were genotyped by pulsed-field gel electrophoresis after *Sma*I macrorestriction, *spa* sequence typing (http://spaserver.ridom.de), and determination of staphylococcal cassette chromosome *mec* (SCC*mec*) type and accessory gene regulator (*agr*) polymorphism (*4*,*5*). Four MRSA isolates were further analyzed by multilocus sequence typing (www.mlst.net). Multiplex PCR was used to test for Panton-Valentine leukocidin, toxic shock syndrome toxin 1, and exfoliatin A and B genes (*6*).

Antimicrobial drug susceptibility was tested by the Vitek2 system (bioMérieux). Multiplex PCR was used to test for resistance genes *tetK*, *tetM*, *aac*(6′)-Ie + *aph*(2″), *ant*(4′)-Ia, *aph*(3′)-IIIa, *ermA,* and *ermC* (*7*–*9*).

Data were analyzed by using Stata 9.2 (Statacorp, College Station, TX, USA). We calculated prevalence of MRSA carriage in humans and 95% confidence intervals (CIs) by using cluster survey analysis. We performed risk factor analysis by using multiple logistic regression for cluster surveys, adjusting for clustering within farms. p values <0.05 were considered significant.

From April through July 2007, veterinarians investigated 50 swine farms. Of 1,500 pigs from 34 farms, 663 (44.2%) carried MRSA (*3*). On 49 of these 50 farms, 127 persons agreed to participate. Nasal (127) and wound (5) swabs showed that 48 (37.8%, 95% CI 25.6%–50.0%) participants carried MRSA and 22 (17%, 95% CI 10.7%–23.9%) carried methicillin-susceptible *S. aureus* (MSSA). One (2%) of the nasal MRSA carriers had a hand lesion infected with MRSA ST398 and treated it with topical antiseptic. Cultures from wounds on 4 other participants were negative for MRSA. Carriers of MRSA and MSSA were found on 25 (51.0%) and 15 (30.6%) farms, respectively. Prevalence of MRSA carriage was 50% for participants on farms with MRSA-colonized pigs versus 3% on farms without colonized pigs (relative risk 16.5, 95% CI 2.4–114.9, p <0.001).

Univariate analysis showed MRSA carriage to be associated with being a farmer or farm co-worker, being male, having regular contact with animals (including goats,

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sheep, dogs, or cats) and, paradoxically, wearing gloves and apron and reporting occasional or regular hand disinfection with an antimicrobial product. Multivariate analysis showed MRSA carriage to be independently associated with MRSA prevalence among pigs at the farm, being a farmer with regular pig contact, reporting regular contact with dogs and horses, and reporting use of protective clothing (apron, gloves, or mask) (Table 1).

The 48 MRSA isolates were nontypeable by *Sma*I; some harbored SCC*mec* type IVa (n = 26), type V (n = 20), or were nontypeable (n = 2), and some exhibited 3 related *spa* types, t011 (n = 45), t034 (n = 2), and t567 (n = 1) (Table 2). Of the *spa* types, 4 representative strains belonged to ST398. Of the strains, 94% were classified into 2 genotypes, t011-SCC*mec* type IVa and t011-SCC*mec* type V, each found on 14 and 10 farms, respectively. In 8 of 11 farms with ≥2 MRSA carriers, all carriers harbored the same *spa*-SCC*mec* genotype. On 17 of 24 farms with MRSA colonization of humans and pigs, both groups carried the same genotype, suggesting animal-to-human transmission (Figure 1). Of MRSA isolates, 40 (83%) were resistant to tetracycline, cotrimoxazole, macrolides-lincosamides, aminoglycosides, and ciprofloxacin (Figure 2). Nearly all strains were susceptible to fusidic acid and mupirocin; all were susceptible to linezolid, rifampin, and glycopeptides. Resistance to aminoglycosides was conferred by the *aac*(6′)–*aph*(2″) gene (n = 23) and the *ant*(4′) gene (n = 9). Resistance to macrolides-lincosamides was mainly mediated by *erm*C gene (n = 24). Tetracycline resistance was encoded by *tet*M and *tet*K genes in 48 (100%) and 23 (50%) isolates, respectively. Resistance profiles were related to clonal types (Table 2).

MSSA isolates belonged to *spa* type t011 or t034 corresponding to ST398 (n = 3) and to 7 PGFE types and 12 *spa* types (n = 19). Genes encoding toxic shock syndrome toxin 1 (n = 4) and exfoliatin A (n = 3) were detected in 7 MSSA isolates. MSSA isolates were susceptible to all antimicrobial drugs except tetracycline (Figure 2).

Conclusions

Human carriage of MRSA was associated with swine colonization with MRSA. Prevalence rate (38%) was higher

Table 1. Risk factors for carriage of methicillin-resistant *Staphylococcus aureus* among 127 persons on 49 pig farms, Belgium, 2007*

Variable	No. carriers	No. noncarriers	aOR (95% CI)	p value
MRSA prevalence among pigs, %				
0	1	32	1	NA
1–49	15	17	50.7 (9.1–283.6)	<0.001
50–84	16	17	90.3 (12.3–664.1)	<0.001
≥85	16	13	85.2 (14.5–501.8)	<0.001
Occupation, pig contact				
No pig contact	4	23	1	NA
Other, ≥1 time/week	9	16	2.7 (0.4–17.5)	0.543
Pig farmer, ≥1 time/week	35	40	14.4 (3.7–55.5)	<0.001
Contact ≥1/week with				
Dogs	16	4	19.8 (4.3–91.2)	<0.001
Horses	7	4	4.8 (1.6–14.2)	0.006
Use of any barrier precaution†	39	59	8.0 (1.8–36.3)	0.008

*Multivariate analysis. aOR, adjusted odds ratio; CI, confidence interval; NA, not applicable.
†Protective clothing, e.g., apron, gloves, mask.

Table 2. Characteristics of 48 methicillin-resistant *Staphylococcus aureus* sequence type 398 isolates from persons on 49 pig farms, Belgium, 2007*

spa type	*spa* repeats	SCC*mec* type	No. isolates	No. farms	AME genes			Methylase genes		Tetracycline resistance genes		Resistance phenotype (>50% isolates)
					aac(6′)–*aph*(2″)	*ant*(4′)	*aph*(3′)	*erm*A	*erm*C	*tet*K	*tet*M	
t011	08–16–02–25–34–24–25	IV	26	14	25	10	0	0	19	5	26	GEN, TOB, ERY, CLI, TET, SXT
		V	19	10	0	0	0	1	3	16	19	CIP, CLI, TET, SXT
t034	08–16–02–25–02–25–34–24–25	V	1	1	0	0	0	0	0	1	1	CIP, CLI, TET, SXT
		NT	1	1	0	1	0	0	0	1	1	TOB, TET
t567	08–02–25–24–25	NT	1	1	0	0	0	0	1	0	1	ERY, CLI, TET

*SCC, staphylococcal cassette chromosome; AME, aminoglycoside modifying enzyme; GEN, gentamicin; TOB, tobramycin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; SXT, cotrimoxazole; CIP, ciprofloxacin; NT, not typeable. All isolates were *agr* group 1.

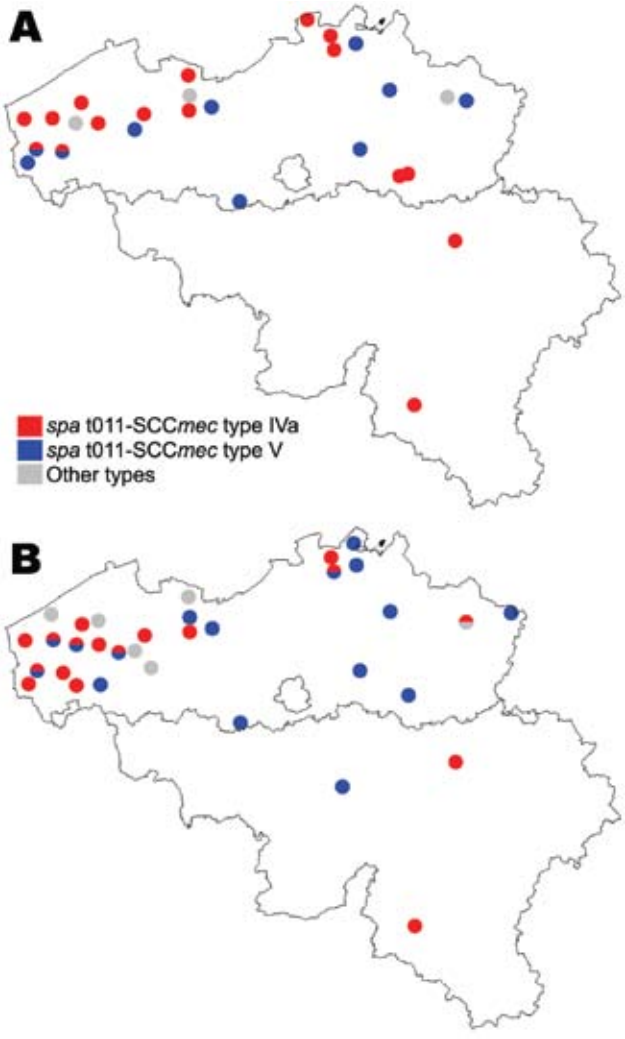

Figure 1. Distribution, by farms, of epidemic methicillin-resistant *Staphylococcus aureus* strains of *spa* type t011-SCC*mec* type IV, t011-SCC*mec* type V, and other types, Belgium, 2007. A) Farm residents and workers; B) Pigs. SCC, staphylococcal cassette chromosome.

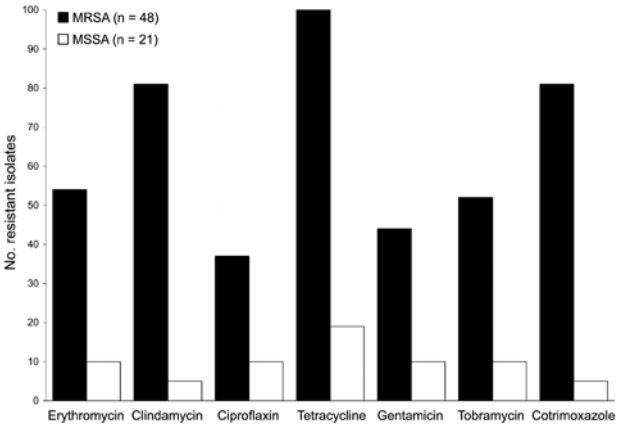

Figure 2. Antimicrobial drug susceptibility of *Staphylococcus aureus* strains recovered from pig farmers, Belgium, 2007. MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-susceptible *S. aureus*.

than that for hospitalized patients or nursing home residents in Belgium (www.nsih.be/surv_mrsa/download_fr.asp). MRSA isolates from farmers belonged to closely related *spa* types corresponding to ST398, which are unrelated to hospital- and community-acquired strains but identical to strains from humans in contact with pigs in other European countries (*1*,*2*,*10*).

Despite the high prevalence of nasal MRSA, active MRSA skin infection was detected infrequently (<1%), within the range described in recent US-based studies (*11*). In a hospital in the Netherlands, a lower attack rate was found for MRSA ST398 than for other MRSA strains (*12*). However, invasive infections caused by MRSA ST398 have been reported, suggesting that this genotype is pathogenic for humans (*2*). In our study, MRSA strains did not harbor exotoxin.

Two MRSA genotypes were predominant. For 70% of farms with multiple MRSA carriers, all strains belonged to the same genotype, suggesting transmission within the farm. Although these strains have been shown to not spread easily in hospitals (*12*), outbreaks of MRSA ST398 in a residential care facility and a hospital probably originated from healthcare workers living on pig farms (*13*,*14*). In contrast with MRSA strains, MSSA isolates in our study showed diverse genotypes that frequently colonize human populations (*4*). MSSA isolates from 3 farmers belonged to the ST398 genotype, which is infrequently reported in humans except in pig farmers with contact with pigs (*4*).

Risk factors for MRSA ST398 carriage included regular contact with pigs but also with horses and dogs (*10*), suggesting that different animals could be MRSA ST398 reservoirs or vectors, at least on pig farms. Protective measures did not seem to reduce the risk of becoming colonized with MRSA; this lack of effectiveness has previously been observed for veterinarians (*15*). This apparent lack of protection should be further investigated to determine routes of transmission other than direct contact with pigs, including airborne transmission and contact with contaminated surfaces and companion animals.

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Eczema Herpeticum and Clinical Criteria for Investigating Smallpox

David A. Boyd, Leonard C. Sperling, and Scott A. Norton

Eczema herpeticum can clinically resemble smallpox. On the basis of the algorithm for rapid evaluation of patients with an acute generalized vesiculopustular rash illness, a patient met criteria for high risk for smallpox. The Tzanck preparation was critical for rapid diagnosis of herpetic infection and exclusion of smallpox.

After the 2001 anthrax bioterrorism incidents, public health officials became concerned about bioterrorist threats of smallpox. The Centers for Disease Control and Prevention (CDC), along with interested partners, developed a clinical algorithm for rapid evaluation of patients with acute generalized vesiculopustular rash illness (AGVPRI) (*1*). In a surveillance system designed to detect an index case of smallpox, high specificity is critical to minimize false-positive reports of a disease that no longer exists in nature (*2*).

CDC's algorithm emphasizes 3 major clinical features of smallpox: febrile prodrome, typical appearance of characteristic lesions, and uniform lesion morphology (Table). The algorithm stratifies AGVPRI cases into high, moderate, and low likelihood of smallpox (*3*). Passive and active surveillance has stratified no case to high risk (*4*). We describe a patient whose illness fulfilled CDC's high-risk criteria for smallpox, although he actually had eczema herpeticum.

The Case

A 45-year-old man with a lifelong history of atopic dermatitis had a year-long unremitting exacerbation for which he had started systemic therapy. After treatment with cyclosporine for several weeks, laboratory abnormalities and nonspecific neurologic signs prompted a switch to methotrexate. Within 4 weeks, he was hospitalized (in an overseas US military hospital) for generalized umbilicated papulopustules accompanied by profound hypothermia, hypotension, and mental status changes. He had large pustules on his trunk, inner thighs, and upper extremities (Figure 1, panel A). Cerebrospinal fluid, obtained because of his obtunded mental status, was unremarkable. The working diagnosis was Sezary syndrome with erythroderma. He was transferred to our intensive care unit with widespread umbilicated pustules and normal mental status. The pustules were deep seated, monomorphic, dome shaped, and firm and were distributed densely on the patient's forearms and abdomen (Figure 1, panels B and C). He showed no enanthem or lesions with an erythematous base. Lesions were abundant on his dorsal hands, but were not palmar. His vital signs were significant only for 100.4ºF temperature. He reportedly had received vaccinia.

At our hospital, his oral temperature fluctuated dramatically, from 89.3ºF to 101.3ºF, with rectal confirmation <95ºF (<35ºC), indicating hypothermia (*5*). He remained normotensive, but his mental status fluctuated.

We believed this smallpox-like eruption most likely resulted from a herpesvirus. We performed a Tzanck preparation, which showed multinucleated giant keratinocytes with nuclear molding and margination (online Appendix Figure, available from www.cdc.gov/EID/content/15/7/1102-appF.htm). A direct fluorescent antibody (DFA) test was positive for varicella zoster virus (VZV). A biopsy specimen showed epithelial necrosis with cellular ballooning and multinucleated giant cells, plus intranuclear inclusion bodies (Figure 2, panels A and B). Subsequently, special immunohistochemical stains were positive for herpes simplex virus (HSV) (Figure 2, panel C), and a viral culture grew HSV type 2. His illness was diagnosed with disseminated HSV concurrent with underlying atopic dermatitis (i.e., eczema herpeticum).

Within minutes of the Tzanck smear evaluation, our patient was given intravenous acyclovir. When cutaneous improvement was evident, he was switched to oral valacyclovir. Within days, his skin lesions largely resolved without conspicuous crusting or scarring, but he remained intermittently hypothermic for several weeks.

Conclusions

This patient was markedly ill on admission and had a distinctive varioliform eruption with lesions in a uniform

Table. Major clinical criteria for smallpox*

Febrile prodrome	Occurring 1–4 days before rash onset; fever ≥101ºF; and ≥1 of the following: prostration, headache, backache, chills, vomiting, or severe abdominal pain.
Classic smallpox lesions	Deep-seated, firm/hard, round, well-circumscribed vesicles or pustules; as they evolve, lesions may become umbilicated or confluent.
Lesions in same stage of development	On any single part of the body (e.g., face or arm); all lesions are in the same stage of development (i.e., all are vesicles or pustules).

*Source: (*3*).

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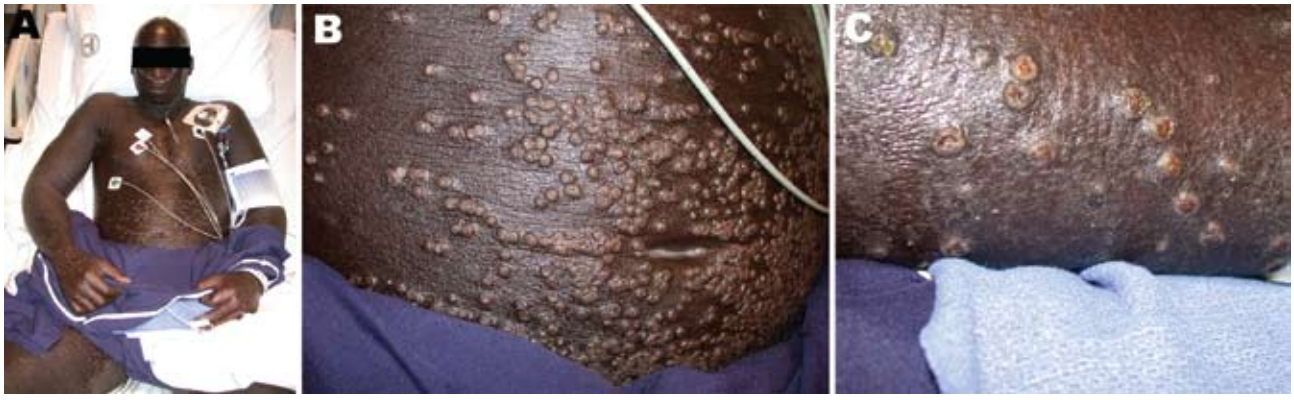

Figure 1. Clinical photographs of the patient. A) Patient with generalized pustules, which were deep seated, monomorphic, dome shaped, and firm and were distributed densely on forearms and abdomen. B) Umbilicated papulopustules. C) Umbilicated papulopustules in the same stage of evolution; no herpetiform clusters or red areolae are seen around the lesions.

stage of evolution. Consequently, smallpox was included in the differential diagnosis. Tzanck preparation promptly confirmed herpetic etiology, but we nevertheless used CDC's algorithm for evaluating AGVPRI, and our patient's illness stratified to high risk.

CDC has 3 major diagnostic criteria to designate a case as high risk for smallpox (Table) (*6*). The first is febrile prodrome, which typically lasts 1–4 days before cutaneous lesions appear and must include ≥1 of the following: prostration, headache, backache, chills, vomiting, or severe abdominal pain. Body temperature must reach ≥101ºF. Although our patient's illness eventually met the fever criterion, his 101ºF temperature occurred only after he began antiviral treatment. He was more often markedly hypothermic during his hospitalization.

Prolonged hypothermia is associated with severe illness (*7*) and is equivalent to fever in determining critical illness (*8*), which we believe satisfies CDC's first major criterion. The second criterion requires classic cutaneous lesions that are deep seated, firm, round, well-circumscribed vesicles or pustules that may become umbilicated or confluent. The third criterion requires the same stage for most cutaneous lesions on an affected area. Our patient's illness met all 3 criteria; however, laboratory tests confirmed herpesvirus infection.

Smallpox was declared eradicated by the World Health Organization in 1977; nevertheless, some health organizations consider this illness a bioterrorism threat. Clinical smallpox typically starts with a prodrome of high fever, headache, myalgia, backache, nausea, vomiting, and diarrhea. An oropharyngeal enanthem is followed by cutaneous eruption of erythematous macules that quickly become papules. The papules evolve over days into vesicles and then pustules, often developing central umbilication. Classic smallpox lesions occur in the same stage of evolution on a body segment, which differentiates it from varicella. Smallpox lesions also tend to start peripherally. Smallpox pustules have been called "pearls of pus" to help distinguish them from the more delicate "dewdrops on rose petals," which describes typical varicella. Histopathologically, cutaneous smallpox lesions may resemble herpetic lesions except that smallpox has intracytoplasmic inclusions (Guarnieri bodies) instead of intranuclear inclusions (Lipschutz bodies) of herpetic lesions. Also, multinucleated giant keratinocytes are uncharacteristic of smallpox (*9*).

Eczema herpeticum, described by Kaposi in 1887, is most common in patients with atopic dermatitis but can occur in other conditions that disrupt epidermal integrity. In eczema herpeticum, lesions are typically monomorphic

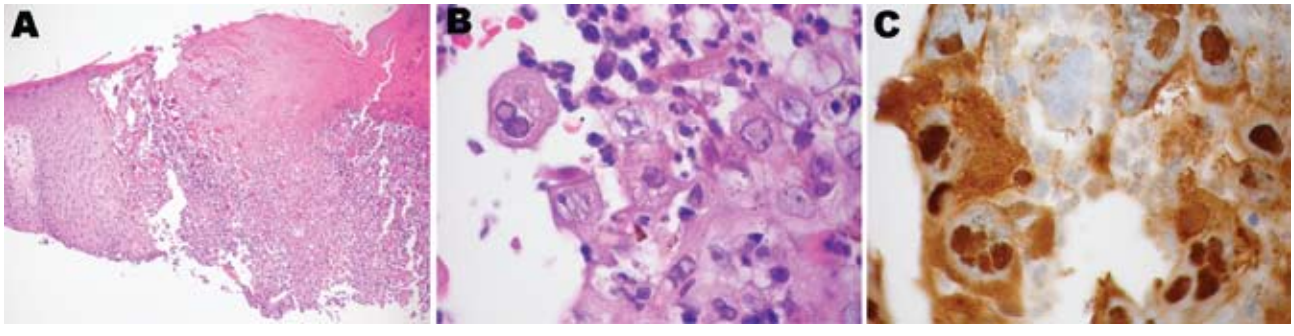

Figure 2. Photomicrographs of the patient's eczema herpeticum. A) Epithelial necrosis with cellular ballooning and multinucleated giant cells. B) Ballooning degeneration of keratinocytes. C) Positive immunohistochemical stain for herpes simplex virus.

vesicles that evolve into pustules (*10*). Fever, malaise, lymphadenopathy, and tender skin may accompany cutaneous eruption (*11*). The histopathologic features noted in our biopsy are classic for herpetic skin lesions.

Fever is a well-recognized sign of infection; however, hypothermia can also signal serious disease, including bacterial sepsis or viral encephalitis (*12*), and may be more dire than fever in severely ill hospitalized patients (*13*). We propose that our patient's hypothermic temperature dysregulation is equivalent to fever, thus serving as a major diagnostic criterion.

This case shows the importance of Tzanck smears to rule out smallpox. When a patient with AGVPRI is evaluated for possible smallpox, rapid laboratory tests are necessary. Viral culture does not yield results quickly enough to avert infection control measures expected with a smallpox case. Indeed, CDC reports 7 incidents when patients with AGVPRI prompted emergency department diversions or hospital closures (*1*). Also, rapid confirmation of nonvariola etiology can help avert public panic, a potential problem in a suspected smallpox outbreak and a probable intended consequence of a terrorist attack.

The Tzanck smear must be performed by someone experienced in using the technique and interpreted by someone who can confidently and correctly distinguish herpesvivus nuclear inclusions from poxvirus cytoplasmic inclusions. DFA for HSV and VZV is relatively rapid, but in our case, the DFA result was positive for VZV, although viral culture and immunohistochemical staining later showed that the patient's infection was due to HSV-2. Had we been unable to confirm a nonvariola etiology, we would have proceeded to poxvirus testing. With no commercially available tests for smallpox, the algorithm advises close coordination among local, state, and federal public health authorities. Some state and federal reference laboratories can provide confirmatory tests, including PCR, for orthopoxviruses such as smallpox and monkeypox. Although not performed in this case, we recommend such testing if a simultaneous infection with an orthopoxvirus cannot be ruled out.

Dr Boyd is a general dermatologist at Naval Hospital Jacksonville, Florida. His interests include military dermatology and teaching residents.

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Rickettsia slovaca and *R. raoultii* in Tick-borne Rickettsioses

Philippe Parola, Clarisse Rovery, Jean Marc Rolain, Philippe Brouqui, Bernard Davoust, and Didier Raoult

Tick-borne lymphadenopathy (TIBOLA), also called *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL), is defined as the association of a tick bite, an inoculation eschar on the scalp, and cervical adenopathies. We identified the etiologic agent for 65% of 86 patients with TIBOLA/DEBONEL as either *Rickettsia slovaca* (49/86, 57%) or *R. raoultii* (7/86, 8%).

In 1968, *Rickettsia slovaca,* a spotted fever group (SFG) rickettsia, was isolated from *Dermacentor marginatus* ticks in the former Czechoslovakia before being detected in *D. marginatus* or *D. reticulatus* ticks throughout Europe (Figure 1) (*1*). In 1997, *R. slovaca* was described as a human pathogen and an agent of tick-borne lymphadenopathy (TIBOLA) (*2*). This syndrome, also called *Dermacentor*-borne necrosis erythema and lymphadenopathy (DEBONEL), is defined as the association of a tick bite, an inoculation eschar on the scalp, and cervical lymphadenopathies (*3*).

Since 1999, several rickettsial genotypes, called DnS14, DnS28, and RpA4, have been detected in *Dermacentor* spp. ticks throughout Europe (Figure 1). Isolates have been obtained and shown to belong to a unique new SFG rickettisia species named *R. raoultii* (*4*). In 2002, *R. raoultii* DNA was detected in a *D. marginatus* tick taken from the scalp of a patient in whom TIBOLA/DEBONEL developed in France (*4*). Moreover, DNA of what is now known to be *R. raoultii* has been found in the blood of 1 patient with TIBOLA/DEBONEL (*5*). The goal of this study was to identify the rickettsial agents in patients with TIBOLA/DEBONEL symptoms and in those who had an isolated tick bite on the scalp.

The Study

We included all patients with TIBOLA/DEBONEL symptoms (Figure 2) and those who had an isolated tick bite on the scalp without any symptoms from whom samples (serum, skin biopsy, or ticks harvested from the scalp) were received at our laboratory from January 2002 through December 2007. Epidemiologic and clinical data were collected retrospectively. The study was approved by the ethics committee of the Medicine School of Marseille under reference 08-008.

Immunoglobulin (Ig) G and IgM titers against rickettsial antigens were estimated by microimmunofluorescent assay; results were verified by Western blot and cross-absorption studies (*3*). Ticks found on persons and skin biopsy specimens were cultured on human embryonic

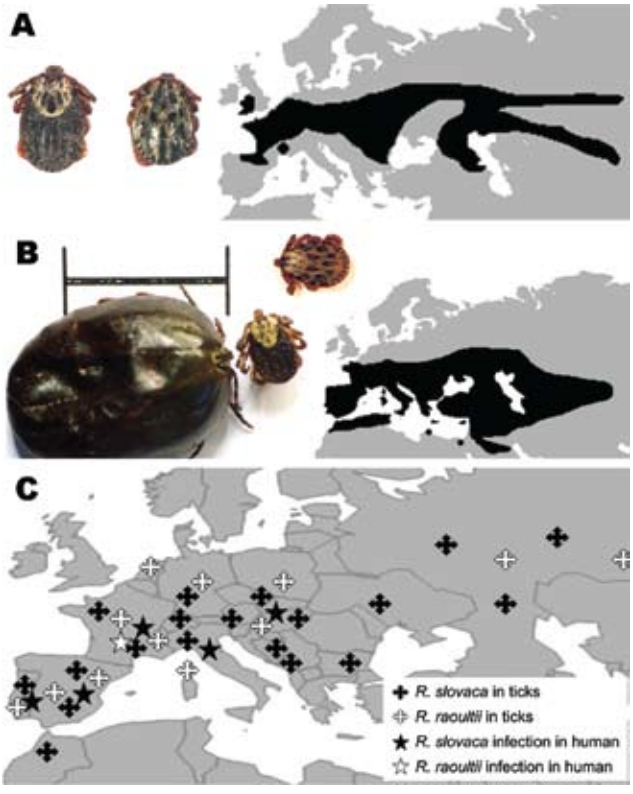

Figure 1. *Dermacentor reticulatus*, the ornate dog tick (A) (female, left; male, right), and *D. marginatus*, the ornate sheep tick (B) (engorged female, left; unfed female, center; male, right; scale bar = 1 cm), and their distribution. *D. marginatus* is most frequently found in Mediterranean areas of Europe with dense bush and tree cover and is common under oak and pine vegetation. It also has a restricted distribution in North Africa, in the cooler and more humid areas associated with the Atlas Mountains. Adults infest large mammals such as sheep, cattle, goats, and wild boars. Larvae and nymphs feed mostly on small mammals and medium sized carnivores. *D. reticulatus* is most frequently found in colder northern areas of western Europe and the former Soviet Union, with high humidity and mild winters. *D. reticulatus* is primarily a tick of dogs and carnivores, but it can be found on ungulates such as sheep, cattle, and horses (*9*). *D. marginatus* and *D. reticulatus* have been suggested as reservoirs of *R. slovaca* and *R. raoultii,* which are maintained in ticks through transstadial and transovarial transmission. Therefore, the geographic distribution of these rickettsiae likely parallels that of *Dermacentor* ticks (C).

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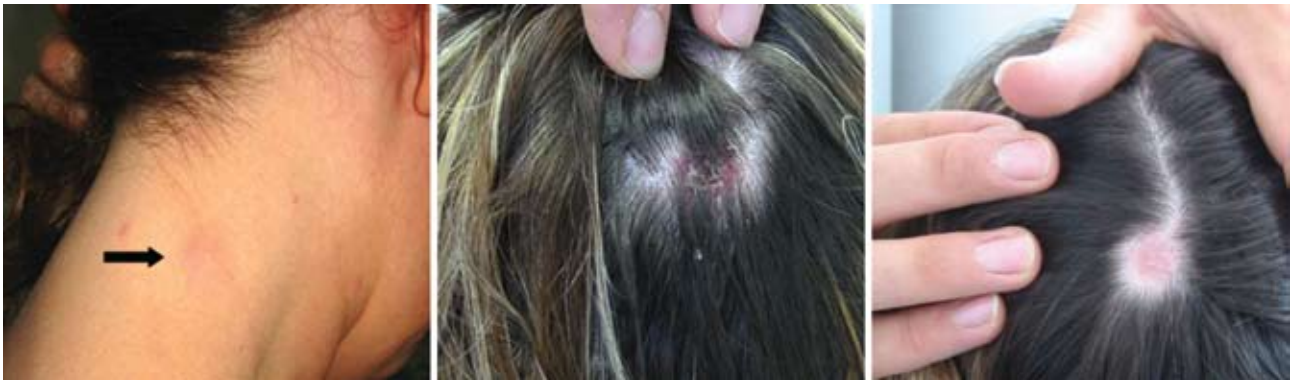

Figure 2. Typical signs of TIBOLA (tick-borne lymphadenopathy)/DEBONEL (*Dermacentor*-borne necrosis erythema and lymphadenophy). Here, infections were caused by *Rickettsia slovaca*, resulting in cervical lymphadenopathy (left panel, arrow), inoculation on the scalp (middle panel), and residual alopecia 4 weeks later (right panel).

lung cells (*6*). These samples were also used to amplify and identify outer membrane protein A–encoding gene fragments of rickettsiae by PCR (*3*). Also, the so-called suicide PCR-assay was used with acute-phase serum samples (*1*).

Among 98 study patients, 86 were classified as TIBOLA/DEBONEL patients. Twelve (12.2%) patients made up the second group with an isolated tick bite. All but 1 patient, who was bitten in Belgium, were bitten in France. Tick bites more frequently occurred from February through May (50/86, 58.1%). Because of results of serologic techniques, we could conclude that 66 (84.6%) of 78 TIBOLA/DEBONEL patients with obtained serum specimens had a recent rickettsial disease. Western blot and cross-adsorption analyses enabled detection of antibodies specifically directed against *R. slovaca* and *R. raoultii* in 34 and 4 patients, respectively (online Appendix Table, available from www.cdc.gov/EID/content/15/7/1105-appT.htm).

Two patients who were infected with *R. slovaca* were found to be co-infected with *Coxiella burnetii* in an acute form of Q fever. Serologic testing was performed in 12 patients with isolated tick bites, and results were negative in all cases. A total of 19 skin biopsy specimens were obtained. Four *R. slovaca* infections were diagnosed by regular PCR, and 3 isolates of this rickettsia were obtained. The suicide PCR on acute-phase serum samples identified 1 additional case of *R. slovaca* infection. Because of molecular tools and culture, 6 patients received the diagnosis of an *R. slovaca* infection, including 5 who did not receive a diagnosis by serologic assays (online Appendix Table). Ticks removed from 28 TIBOLA/DEBONEL patients consisted of 23 *D. marginatus* (88.4%), 2 *Dermacentor* spp., 1 *Haemaphysalis punctata*, and 2 that were not identified. Overall, the tick studies enabled us to suggest the diagnosis of *R. slovaca* and *R. raoultii* infections in 10 and 3 patients, respectively, whose conditions were not diagnosed with previous tests (online Appendix Table).

All DNA sequences obtained showed 100% identity with *R. raoultii* or *R. slovaca*, excluding the coexistence of several rickettsiae in the corresponding samples. According to our investigations, 49 (57%) of 86 patients with TIBOLA/DEBONEL had probable or certain *R. slovaca* infections, and 7 (8%) of 86 had probable *R. raoultii* infections. The characteristics of these patients are shown in the Table.

Conclusions

We report 86 patients with TIBOLA/DEBONEL; this group includes 14 patients whose conditions had been preliminarily reported (*7*). We also describe several cases caused by the emerging pathogen *R. raoultii* (*4*), including patients with indirect molecular evidence of infection because the pathogen was detected in the ticks that had bitten them. Original findings also include facial edema as a new clinical feature in TIBOLA/DEBONEL, and the report of the second patient co-infected with *R. slovaca* and *C. burnetii* (*8*). Because acute Q fever, a worldwide zoonosis, may be asymptomatic, we recommend that patients infected with tick-borne pathogens also undergo testing for concurrent infections with *C. burnetii*.

No TIBOLA/DEBONEL cases were recorded during the warmest summer months; peak incidence occurred during March–May and during September–November, linked with the activity of *Dermacentor* ticks in Europe (Figure 1) (*9*). However, to date, we have no explanation for the finding that children and women are at higher risk for TIBOLA/DEBONEL or why *D. marginatus* and *D. reticulatus* ticks prefer to bite persons on the scalp. A possible explanation could be that *Dermacentor* ticks usually bite hairy domestic and wild animals and the longer hair of women and children may attract them.

One of the most remarkable findings of this work is the proportional importance of *R. slovaca* in TIBOLA/DEBONEL patients, compared with *R. raoultii*. In 2006, Ibarra

Table. Characteristics of TIBOLA/DEBONEL patients with certain or probable *Rickettsia slovaca* infection compared with patients with certain or probable *R. raoultii* infection*

Characteristic	TIBOLA/DEBONEL patients, n = 86		
	No. *R. slovaca* infections (%), n = 49†‡	No. *R. raoultii* infections (%), n = 7†	p value
Female sex	33/49 (67)	7/7 (100)	0.04
Mean age, y	32	32	0.90
Age ≤12 y	20/49 (41)	3/7 (43)	0.46
Hiking or recreational activities such as a walk in the forest	21/28 (75)	4/5 (80)	0.44
Fever§	21/39 (54)	4/5 (80)	0.27
Painful eschar	14/22 (64)	3/3 (100)	0.30
Painful adenopathies	18/26 (69)	5/5 (100)	0.20
Face edema	6/31 (19)	2/5 (40)	0.30
Rash	7/30 (23)	1/5 (20)	0.68
Headache	16/30 (53)	4/4 (100)	0.10
Alopecia	16/27 (59)	0/4	0.09
Asthenia	23/33 (70)	5/5 (100)	0.20
Prolonged asthenia¶	10/29 (35)	2/4 (50)	0.46
Chronic asthenia#	4/28 (14)	1/4 (25)	0.51

*Certain cases were those with positive culture, PCR, or suicide PCR results in blood or skin biopsy samples or with lymph node aspirates. Probable cases were those with identification by PCR and sequencing of the corresponding *Rickettsia* spp. in ticks, Western blot results demonstrating *R. slovaca*– or *R. raoultii*–specific antibodies, or a cross-absorption assay demonstrating specific antibodies against *R. slovaca* or *R. raoultii.* TIBOLA, tick-borne lymphadenopathy; DEBONEL, *Dermacentor*-borne necrosis erythema and lymphadenopathy.
†Denominators indicate the number of patients for whom the criterion was available.
‡Includes 14 patients previously reported (*7*).
§Temperature >37°5C.
¶Self-reported, persistent asthenia of 1 to 6 months.
#Self-reported persistent or relapsing asthenia of >6 consecutive months.

et al. reported on 14 persons in Spain who had a *D. marginatus* tick attached to the scalp (*10*). All ticks were found to be infected by rickettsiae: 8 (58%) were infected by *R. slovaca,* and 6 (42%) by *R. raoultii*. In 10 of the patients, TIBOLA/DEBONEL symptoms developed, including in all 8 of the patients who had been bitten by a tick infected by *R. slovaca* and in 2 of the 6 patients who had been bitten by a tick infected by *R. raoultii*. *R. slovaca* was more significantly associated with TIBOLA/DEBONEL patients than was *R. raoultii* ($p<0.05$) (*10*). Here, focusing on the studies of ticks removed from TIBOLA/DEBONEL patients, we found that 12 of 19 ticks harbored *R. slovaca*, whereas only 3 of 19 harbored *R. raoultii* ($p = 0.047$). In the patients with asymptomatic tick bites, from whom 9 ticks were obtained, all ticks positive by PCR harbored *R. raoultii*.

Moreover, *R. raoultii* seems to be more highly prevalent in *D. marginatus* and *D. reticulatus* ticks in nature than is *R. slovaca*. Although comparing field surveys of ticks is difficult because of the sampling methods, the sizes of the samples, and the potential PCR inhibitors, *R. raoultii* has been more frequently detected in *D. marginatus* ticks than has *R. slovaca*. In southeastern Spain, 73% of 101 *D. marginatus* ticks were infected by *R. raoultii* and 27% by *R. slovaca* (*11*). Similar differences have been shown in Germany, Portugal, the Netherlands, and Spain (*12–15*). Although interpreting these data definitively is difficult, the recurrence of similar published results by different teams suggests that exposure to *R. raoultii* through the bite of a *Dermacentor* spp. tick is likely more frequent than exposure to *R. slovaca*. However, more cases of *R. slovaca* infection have been recorded, which suggests that *R. raoultii* is less pathogenic.

TIBOLA/DEBONEL is a newly recognized disease, and its incidence is likely underestimated. In our laboratory, TIBOLA/DEBONEL is the most frequently reported rickettsial disease, except during the dry summer period. Doxycycline remains the treatment of choice, with new macrolides as alternative treatments (*1*). Although we report 6 more cases of *R. raoultii* infection in addition to the 2 recently reported (*4,5*), this *Dermacentor*-borne rickettsia seems to be less pathogenic than *R. slovaca*.

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Latent Tuberculosis and Active Tuberculosis Disease Rates among the Homeless, New York, New York, USA, 1992–2006

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We conducted a retrospective study to examine trends in latent tuberculosis infection (LTBI) and TB disease rates among homeless persons in shelters in New York, NY, 1992–2006. Although TB case rates fell from 1,502/100,000 population to 0, a 31% LTBI rate in 2006 shows the value of identifying and treating TB in the homeless.

Tuberculosis skin testing (TST) at homeless shelters and drop-in centers is a standard component of tuberculosis (TB) transmission control. Since 1969, St. Vincent's Hospital has cared for homeless persons in New York City (NYC) (*1*), and 37 shelters and drop-in centers were served in 2006. We report latent tuberculosis infection (LTBI) and TB disease data from the 8 largest of these sites from January 1992 through June 2006. TB screening began at the largest (site 1) in January 1992 and at the others in 1997 through 1999 (Table 1). Because the NYC homeless population is diverse, the populations served by shelters have qualitatively and quantitatively different LTBI risk factors. Sites 1–3 are men's shelters, site 8 is a women's drop-in center, and sites 4–7 are drop-in centers for both men and women. At the sites, medical teams assess persons' TB status using a standard questionnaire and, when indicated, TST. The questionnaire was designed to elicit TB history, prior skin test results, LTBI treatment, and risk factors, including those for progression to TB disease, such as HIV infection. During the time encompassed by this study, St. Vincent's Hospital initiated 32,108 TB evaluations of homeless persons at these 8 sites; 28,835 (89.8%) were completed.

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The Study

We designed and conducted a retrospective, descriptive study to examine trends in LTBI and TB disease rates among homeless persons at the selected sites. Our dataset was limited to the following categories: age, sex, race/ethnicity, prior TST status, HIV test status, and LTBI treatment history. Those with no previous TST, prior negative tests, or unclear prior test results were offered a TB evaluation. TST was not offered to those with a convincing past history of TB or a documented or convincing history of a previous positive TST result (explanation in online Technical Appendix, available from www.cdc.gov/EID/content/15/7/1109-Techapp.pdf). We classified each person as follows: TST negative, TST positive as tested, TST positive by history, TB disease, and noncompliant with skin testing reading.

The number of single adult homeless persons identified by the yearly census of the New York City Department of Homeless Services ranged from 27,846 in 1992 to 29,348 in 2005 (*2*), while the number and proportion of TST screenings by our program increased from 972 (3% of New York City total) in 1992 to 4,093 (13.9%) in 2005, the last complete year of our data.

Among the 32,108 attempted TB screenings, 28,835 persons (89.8%) completed the screening process; 11,385 had positive TST results or history (Table 2). We stratified TST data from all sites and compared those from site 1 (58.1% of observations) to those from sites 2–8 combined, for all years. Using a nonparametric test for trend across ordered groups (developed by Cuzick [*3*] as an extension of the Wilcoxon rank-sum test), we found the 4 testing groups each had statistically significant negative trends by year: site 1 test only, $z = -3.64$, $p<0.001$; site 1 test and history, $z = -3.35$, $p = 0.001$; sites 2–8 test only, $z = -2.91$, $p = 0.004$; sites 2–8 test and history, $z = -2.95$, $p = 0.003$ (Stata 10.1 manual: nptrend test) (*4*). The statistically significant decreasing trends for the TST and TST positive by history groups for both site 1 and the other sites combined, from the inception of the data collection, are shown in Figure 1.

Overall, when the categories of administered TST and TST positivity by history were combined as indicators of LTBI, results decreased from 58.1% to 30.9% (1992–2006) at site 1 and from 57.3% to 30.8% at sites 2–8 combined (1997–2006) (online Technical Appendix Table 1). Since few persons with a history of TST provided documentation, we examined LTBI rates among those persons actually tested. By this criterion, TST positivity decreased from 40.1% to 9.8% at site 1 (1992–2006) and from 30.4% to 13.3% at sites 2–8 combined (1997–2006).

TB disease rates at all 8 sites combined (Figure 2) also decreased in the screened population over the study period. Sixty-three persons with TB disease were identified from January 1992 through June 2006. In 1992, the calculated

Table 1. Number of tuberculin skin test screenings, by site, New York, NY, USA, January 1992–June 2006

	Shelters			Drop-in centers					
Year	1	2	3	4	5	6	7	8	Total
1992	972	–	–	–	–	–	–	–	972
1993	802	–	–	–	–	–	–	–	802
1994	822	–	–	–	–	–	–	–	822
1995	967	–	–	–	–	–	–	–	967
1996	1,419	–	–	–	–	–	–	–	1,419
1997	1,320	141	45	–	–	–	–	–	1,506
1998	1,208	533	160	2	6	6	–	3	1,918
1999	1,110	550	172	172	167	112	131	151	2,565
2000	1,141	628	114	187	173	166	113	167	2,689
2001	1,153	740	243	180	169	159	91	86	2,821
2002	1,147	717	428	157	141	150	117	85	2,942
2003	1,441	782	441	132	134	96	122	46	3,194
2004	1,548	1,065	209	126	132	86	93	87	3,346
2005	1,958	1,443	78	127	182	145	121	39	4,093
2006*	1,016	722	17	66	91	85	51	4	2,052
Total	18,024	7,321	1,907	1,149	1,195	1,005	839	668	32,108

*January–June.

case rate was 1,502/100,000 population compared with 171/100,000 in 2004. These rates are far greater than for the general New York City population (13.0/100,000) and the United States population (4.4/100,000) in 2007 (*5*). From January 2005 through June 2006, no cases were identified. Treatment history was included in the TB questionnaire starting in 1996. The percentage of persons with a history of positive TST and who received ≥6 months of treatment trended upward, from 50.0% in 1996 through 1997 to 84.2% in 2006 (online Technical Appendix Figure).

Using logistic regression, we modeled the risk factors (online Technical Appendix Tables 1–4), and the probability of being TB positive (by test or history) and of not having the TB test read. For TB positivity, the adjusted odds ratio (OR) for each age group compared with that for the youngest age group was significant and increased linearly with age. In general, the OR increased by a factor of 2 for each advancing age group (online Technical Appendix Table 3). Regarding compliance in TST reading, being in an older group was protective (online Technical Appendix Table 3).

Table 2. Results of TB screenings, New York, NY, USA, January 1992–June 2006*

TB screening status	No. (%)
Noncompliant with skin test reading	3,273 (NA)†
TST–	17,368 (60.4)
TST+	3,597 (12.5)
TST+ by history	7,788 (27.1)
Active TB	63 (<1.0)
Anergic‡	19 (<1.0)
Total completing evaluation	28,835 (100.0)

*TB, tuberculosis; TST, tuberculin skin testing; NA, not applicable.
†The denominator includes all those persons for whom TST status became known. Those noncompliant with TST interpretation have not been included. Thus, in online Technical Appendix Table 4 (available from www.cdc.gov/EID/content/15/7/1109-Techapp.pdf), the percentage of such persons is NA.
‡No reaction to TST, mumps virus, *Candida* spp.

We were able to assess changes in HIV-positivity rates from January 2001 through June 2006. The percentage fluctuated between 2.1% and 3.1%. The stage of HIV disease (from asymptomatic HIV-antibody positive to AIDS) was not recorded. For comparison, during this period in New York City, the number of persons who received a diagnosis of AIDS each year decreased from 5,616 to 3,672 (*6*). Because the percentage of HIV antibody positivity among those screened was essentially constant during the study period, we believe that the decreasing LTBI rates (represented by TST positive as tested) are real and not due to increasing numbers of persons with advanced HIV disease who are unable to mount an appropriate skin test response.

Our acceptance of a compelling history of prior positive TST rather than retesting may have resulted in inaccurate reporting of LTBI rates. However, we decided to accept compelling histories on the basis of decades of clinical experience in caring for homeless persons, experience in completing forms in the same way over the study years, and by a desire to do no harm. Our population is not a random

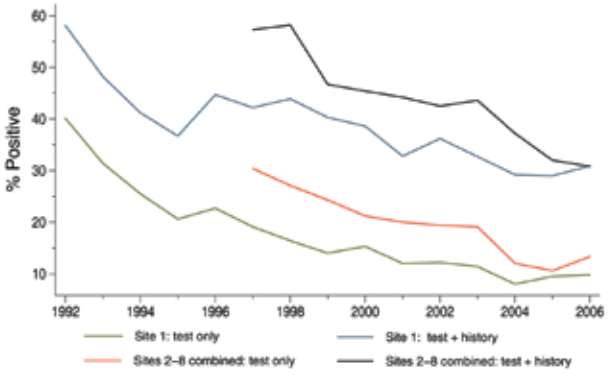
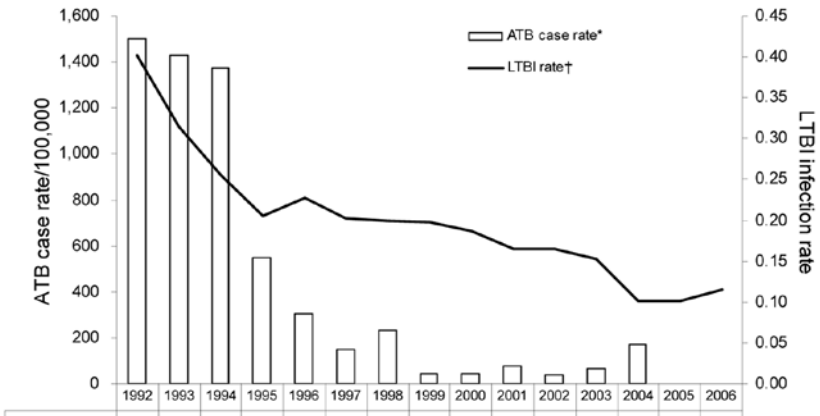


Figure 1. Tuberculin skin test positivity by site and year, New York, NY, USA, January 1992–June 2006.



	1992	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006
ATB case rate*	1,502	1,429	1,372	550	306	148	235	44	42	80	37	67	171	–	–
LTBI rate†	0.40	0.31	0.25	0.21	0.23	0.20	0.20	0.20	0.19	0.17	0.17	0.15	0.10	0.10	0.12

Figure 2. Tuberculosis disease case rates (per 100,000) and infection rates (as tested) by year, New York, NY, USA, 1992–2006. ATB, active tuberculosis; LTBI, latent tuberculosis infection.

sample and may not represent the total homeless population within the city, raising concern about the generalizability of our findings. Also, because the results of all TST screenings were included in our analysis, persons who were screened multiple times are overrepresented. Of 32,108 attempted screenings, 4,724 (14.7%) persons received >1 evaluation. Also, the number of screenings at each site over the years varied, depending on changing requirements of the shelter program.

In the past 2 decades, major public health efforts have been made to evaluate and treat persons with both TB disease and LTBI. Our analysis offers evidence that these attempts to control TB in the homeless population have been beneficial (*7*–*9*).

Although case rates of TB disease are carefully measured by public health authorities throughout the United States, this is not true for LTBI rates. Therefore, we cannot directly compare the rates of LTBI among these homeless persons to those among the general population. However, the rates of LTBI and TB disease observed in this study may serve as a valuable resource for TB control planning.

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Chinese-like Strain of Porcine Epidemic Diarrhea Virus, Thailand

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Since late 2007, several outbreaks of porcine epidemic diarrhea virus (PEDV) infection have emerged in Thailand. Phylogenetic analysis places all Thai PEDV isolates during the outbreaks in the same clade as the Chinese strain JS-2004-2. This new genotype PEDV is prevailing and currently causing sporadic outbreaks in Thailand.

Porcine epidemic diarrhea virus (PEDV), first recognized in 1977 (*1*), is an enveloped, single-stranded RNA virus belonging to the family *Coronaviridae*. The PEDV genome contains genes for the following proteins: pol1 (P1), spike (S) (180–220 kDa), envelope (E), membrane (M) (27–32 kDa), and nucleocapsid (N) (55–58 kDa) (*2*). The M protein is a structural membrane glycoprotein, which plays an important role in the assembly process; the S surface glycoprotein harbors the specific host cell receptor binding sites (*3*).

During late 2007, the PED outbreak appeared first in Nakornpathom province before spreading throughout the country. Pig losses from the recent PED outbreaks were extensive. Obvious clinical signs were severe diarrhea (Figure 1, panel A) and dehydration with milk curd vomitus in suckling piglets. Most of the affected farms reported the disease first in farrowing barns and subsequently lost 100% of newborn piglets. Pigs of all ages were affected and exhibited degrees of diarrhea and inappetite, which varied by their ages. Boars and sows had mild diarrhea and anorexia for a few days and recovered within a week. In piglets that died, the small intestinal wall was congested and intestinal contents were watery with undigested milk curd (Figure 1, panel B). Segmental enteritis was indicated by segmental disappearance of intestinal lacteal caused by malabsorption in affected intestinal parts (Figure 1, panels C and D). Atrophic enteritis, characterized by blunting of the intestinal villi and sloughing of intestinal epithelium, occurred in all affected piglets (Figure 2, panel A). Immunohistochemical tests, performed by using monoclonal anti-PEDV S protein (JBT Biotechnology Laboratory, Seoul, South Korea), demonstrated dark brown staining in intestinal epithelial cells (Figure 2, panel B). Massive feedback of piglet feces and minced piglet guts to gestating sows was recommended by local veterinary practitioners to prime the sow's immune response and pass protective immunity to the piglets. At affected farms, the outbreak lasted <3 weeks.

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The Study

Samples from 8 provinces (24 farms) in Thailand from December 2007 through March 2008 were submitted to the veterinary diagnostic laboratories of Kasetsart University and Chulalongkorn University. A total of 33 porcine samples were confirmed as positive for PEDV by reverse transcription–PCR (RT-PCR) (*4*) before virus isolation (Table). Published primers (*5*) were used for generating the PEDV 651-bp partial S gene. Primers were designed to amplify the PEDV M gene and yielded the amplified product of 715 bp on the basis of CV777 and Br1/87. Products were purified by using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and were sequenced by 1st BASE Pte Ltd (Singapore).

Nucleotide and deduced amino acid sequences of the 33 PEDV isolates were aligned, edited, and analyzed with ClustalX version 1.83, Bioedit version 7.0.5.2, and MegAlign software (DNAStar Inc., Madison, WI, USA), respectively. Phylogenetic trees were generated by using partial S and full-length M genes, including the deduced amino acid sequences with selected reference PEDV strains, by applying the Jotun Hein method in the MegAlign software. To assess the relative support for each clade, bootstrap values were calculated from 1,000 replicate analyses.

The M gene sequence analysis of 31 PEDV isolates obtained in Thailand indicated that the nucleotide sequence of the entire M gene was highly conserved. All recent PEDV isolates in Thailand had 99.3%–100% nucleotide homology. The lowest sequence identity (96.5%) was with the Chinese strain, EF185992/LZC, and the highest sequence identity (99.2%–99.7%) was with the Chinese strain, JS-2004-2, and concurrent isolates from the National Institute of Animal Health, Thailand, M_NIAH 07-08 (data not shown).

All 33 PEDV isolates had 97.0%–98.8% DNA sequence identities of the S gene with each other. Our findings demonstrated that the recent PEDV isolates in Thailand were genetically diverse in their S genes either within

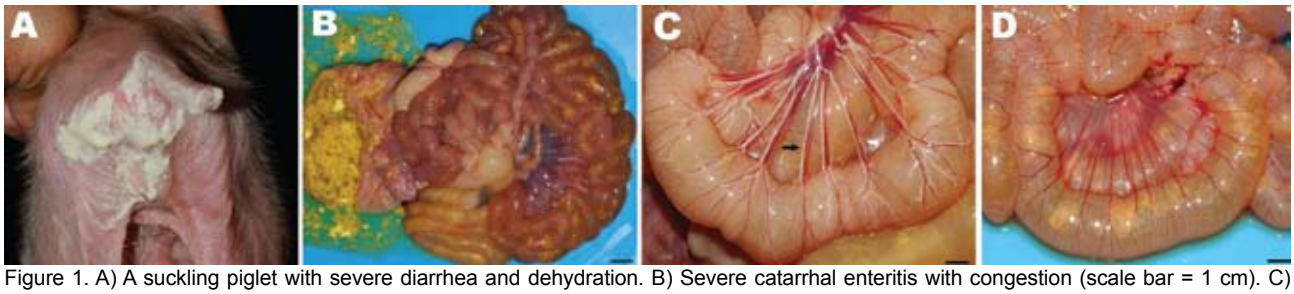

Figure 1. A) A suckling piglet with severe diarrhea and dehydration. B) Severe catarrhal enteritis with congestion (scale bar = 1 cm). C) Intestinal lacteals (arrow) grossly demonstrating normal absorption capacity of the intestinal villi in a normal piglet (scale bar = 0.5 cm). D) Disappearance of intestinal lacteals demonstrating malabsorption syndrome of the intestinal villi in the infected piglet (scale bar = 0.5 cm).

their group or with the reference strains. These point mutations may lead to genetic diversity among these isolates. The lowest sequence identity (95.7%) was with the Korean strain, Chinju99. Similar to the M gene results, the highest sequence identity of the S gene (98.6%) was with the Chinese strain, JS-2004-2 (data not shown).

Three major clusters based on the phylogenetic relation of the nucleotide sequences of the M gene (online Appendix Figure panel A, available from http://www.cdc.gov/EID/content/15/7/1112-appF.htm) were detected. The first cluster comprised all Thai isolates in 2007–2008 and 3 Chinese strains (JS-2004-2, LJB/03, and QH). The second cluster consisted of 2 Korean strains (KPEDV-9 and Chinju99), 1 Japanese strain (Jme2), 2 Chinese isolates (HN-XYYYP-2007 and YM 2007), and a previous Thai isolate in 2004 (M_NIAH_04). The third cluster contained Br1/87 (CV777), 2 Russian isolates, and 1 Chinese strain (LZC).

On the basis of the phylogenetic relation of the nucleotide sequences of the partial S gene, 3 groups were identified (online Appendix Figure panel B). Group 1 comprised all recent Thai PEDV and the Chinese PEDV strains isolated in 2003–2004 (JS-2004-2 and LJB/03). Notably, a Thai isolate, 08NP04, was very similar to JS-2004-2. Group 2 comprised Br1/87 (LZC) and CV777. Group 3 consisted of the Korean isolates (Spk1 and Chinju99).

The recent Thai PEDV strain was closely related to the isolates from China, JS-2004-2 and LJB/03. There was no insertion or deletion in the M gene of the recent Thai isolates except for minor point mutations. M_NIAH/04, isolated in 2004 in Thailand, had a slightly different nucleotide sequence from the recent Thai isolates (R. Thanawongnuwech, unpub. data).

Our results indicated that the recent Thai PEDV isolates clustered in the same group were highly homologous with the Chinese strains, JS-2004-2 and LJB/03. They were responsible for the recent PED outbreak in Thailand and able to produce pathologic effects similar to the Chinese isolates (*6*). Notably, 08NP04, isolated 4 months after the first outbreak, had the highest identity to JS-2004-2. Also, 08NP04 and 07NP01 (the first isolates in 2007) originated in the same geographic area. The Chinese-like strain of the virus might have gained entry into Thailand via unknown routes as early as December 2007. In addition, rendering trucks traveling from farm to farm might have encouraged widespread transmission of the disease. Structural differences in the partial S gene could help elucidate pathogen-

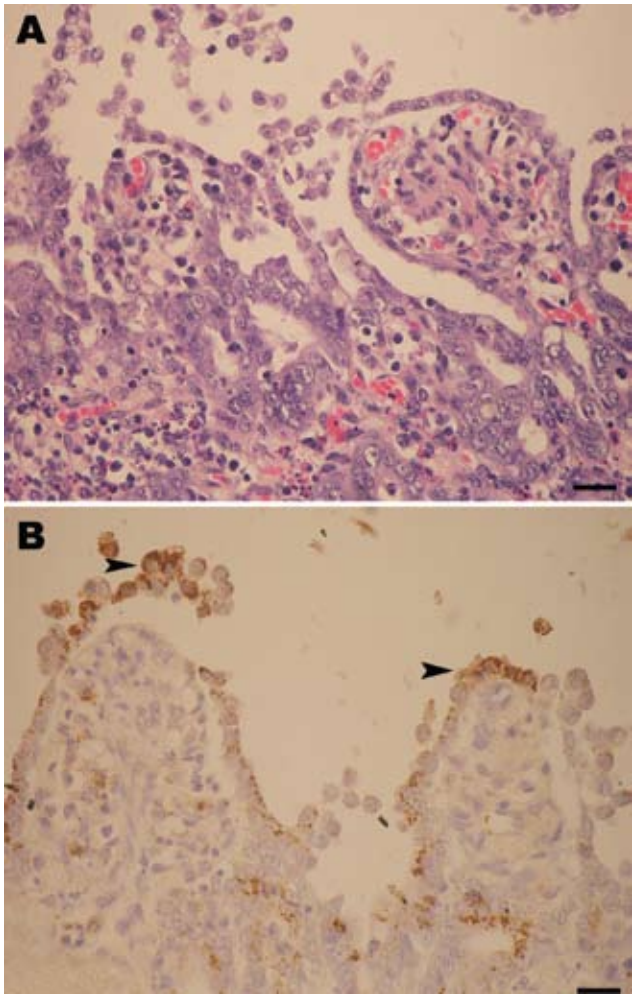

Figure 2. A) Marked shortening and blunting of the intestinal villi (scale bar = 25 μm). B) Intestinal epithelial cells expressing porcine epidemic diarrhea virus antigen (arrowheads) in the cytoplasm (colon), visible as brown staining (scale bar = 25 μm).

Table. Thirty-three PEDV isolates obtained from 8 provinces in Thailand during outbreaks, 2007–2008*

Isolate no.	Isolate name	Date isolated	Geographic origin	GenBank accession nos., M/S gene
1	07NP01	2007 Dec	Nakornpathom (W)	FJ196165/FJ196196
2	08CB01	2008 Jan	Chonburi (E)	FJ196166/FJ196197
3	08RB01	2008 Jan	Ratchaburi (W)	FJ196182/FJ196213
4	08RB02	2008 Jan	Ratchaburi (W)	FJ196183/FJ196214
5	08CB02	2008 Jan	Chonburi (E)	FJ196167/FJ196198
6	08RB03	2008 Jan	Ratchaburi (W)	FJ196184/FJ196215
7	08NP02	2008 Jan	Nakornpathom (W)	FJ196173/FJ196204
8	08NP03	2008 Jan	Nakornpathom (W)	FJ196174/FJ196205
9	08PB01	2008 Jan	Pretchaburi (S)	FJ196180/FJ196211
10	08NP04	2008 Jan	Nakornpathom (W)	FJ196175/FJ196206
11	08CB03	2008 Jan	Chonburi (E)	FJ196168/FJ196199
12	08NP05	2008 Feb	Nakornpathom (W)	FJ196176/FJ196207
13	08RB04	2008 Feb	Ratchaburi (W)	FJ196185/FJ196216
14	08CB04	2008 Feb	Chonburi (E)	FJ196169/FJ196200
15	08RB05	2008 Feb	Ratchaburi (W)	FJ196186/FJ196216
16	08RB06	2008 Feb	Ratchaburi (W)	FJ196187/FJ196217
17	08CB05	2008 Mar	Chonburi (E)	FJ196170/FJ196201
18	08NP06	2008 Mar	Nakornpathom (W)	FJ196177/FJ196208
19	08NP07	2008 Mar	Nakornpathom (W)	FJ196178/FJ196209
20	08UB01	2008 Mar	Ubon Ratchathani (NE)	FJ196189/FJ196220
21	08CC01	2008 Mar	Chachoengsao (E)	FJ196172/FJ196203
22	08CB06	2008 Mar	Chonburi (E)	FJ196171/FJ196202
23	08RB07	2008 Mar	Ratchaburi (W)	FJ196188/FJ196219
24	08NP08	2008 Mar	Nakornpathom (W)	FJ196179/FJ196210
25	08PC01	2008 Mar	Prachinburi (E)	FJ196181/FJ196212
26	KU01CB08	2008 Jan	Chonburi (E)	FJ196190/FJ196221
27	KU02NK08	2008 Feb	Nongkhai (NE)	–/FJ196222
28	KU03CB08	2008 Feb	Chonburi (E)	FJ196191/FJ196223
29	KU04RB08	2008 Feb	Ratchaburi (W)	FJ196192/FJ196224
30	KU05CB08	2008 Mar	Chonburi (E)	FJ196193/FJ196225
31	KU06RB08	2008 Mar	Ratchaburi (W)	FJ196194/FJ196226
32	KU07RB08	2008 Mar	Ratchaburi (W)	FJ196195/FJ196227
33	KU08RB08	2008 Mar	Ratchaburi (W)	–/FJ196228

*PEDV, porcine epidemic diarrhea virus; E, eastern, W, western, NE, northeastern; S, southern.

esis and antigenic structures of the recent PEDV isolates because S glycoproteins are responsible for inducing the virus neutralizing antibodies and known to be highly conserved in PEDV strains (*7*). Continuing investigation of PEDV isolates will contribute to the prevention and control of this virus.

Conclusions

The phylogenetic relationship of the Thai PEDV strain indicated that the recent Thai PEDV isolates differed genetically from previous Thai isolates. Despite precautions, sporadic outbreaks continue to occur. In addition, disease transmission frequently occurs due to the purchase of new stock with improper gilt acclimatization and biosecurity. Immunity induced through vaccination, currently unavailable in Thailand, does not provide lifelong protection from this virus. However, vaccination is recommended to encourage specific immunity to PEDV in all stock when an acute outbreak occurs. Undoubtedly, effective biosecurity is a key management tool for PED prevention and control. Our data suggested that all recent Thai PEDV isolates are genetically similar to the Chinese isolates identified in 2004. Further analysis of the entire S gene of PEDV and of other isolates in neighboring countries is needed to show the molecular epidemiology of the Chinese-like strain.

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Epidemiology of Human T-cell Lymphotropic Virus Type 1 Infection in Blood Donors, Israel

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The prevalence of infection with human T-cell lymphotropic virus type 1 (HTLV-1) in blood donors from Israel is 1 infection/100,000 persons. In donors originating from Eastern Europe, the Middle East, and Latin America, prevalences are 7.7, 14.6, and 20.4, respectively. HTLV-1 prevalence may be high outside areas where HTLV-1 previously was known to be endemic.

Human T-cell lymphotropic virus type 1 (HTLV-1) is prevalent mostly in Japan, Africa, the Caribbean Islands, and South America (*1,2*). Known HTLV-1 modes of transmission include vertical transmission (predominantly through breastfeeding), transverse transmission (sexual intercourse), transfusion of infected cellular blood products, and sharing of needles and syringes (*1,2*). Because of reports of HTLV-1–associated diseases in Mashhadi Jews, the Israeli national blood services, Magen David Adom, began screening all blood units for HTLV-1 antibodies in 1995. However, the prevalence of HTLV-1 infection in the general Israeli population has not yet been defined.

Israel is an immigration state, providing a unique opportunity to examine the prevalence of HTLV-1 infection according to donors' countries of origin. This information may reflect the distribution of HTLV-1 within the respective countries of origin, some of which have not had HTLV-1 serosurveys performed.

The Study

Blood donation in Israel is voluntary and does not involve any monetary benefit. Using records from Magen David Adom, we registered age, sex, country of birth, and maternal and paternal countries of birth once for each donor, regardless of the number of blood units donated.

From 1995 through 1998, donors were screened for antibodies against HTLV-1 and HTLV-2 by standard ELISA (Abbott HTLV-1/HTLV-2 enzyme immunoassay; Abbott Laboratories, Abbott Park, IL, USA). Since 1998, testing has been performed by chemiluminescent immunoassay with the PRISM assay (Abbott Laboratories). The confirmatory assay was Western blot HTLV Blot 2.4 (Genelabs Diagnostics, Singapore Science Park, Singapore).

On the basis of virus transmission modes, we developed an algorithm for identifying the ethnic origin of both HTLV-1–positive and HTLV-1–negative blood donors (Figure 1). We considered infection to be acquired in Israel when the donor and both parents were born in Israel. We considered infection to be acquired outside Israel when the donor or 1 parent was born outside Israel. When the donor was born in Israel and the mother was born outside Israel, country of origin was considered the mother's country of birth. When the donor and the mother were born in Israel, but the father was born outside Israel, country of origin was considered the father's country of birth. Detailed classification of geographic origin of blood donors (both HTLV-1 positive and HTLV-1 negative) is given in the online Technical Appendix (available from www.cdc.gov/EID/content/15/7/1116-Techapp.pdf).

Data were analyzed by using Microsoft Access (Microsoft, Redmond, WA, USA) and Epi Info (Centers for Disease Control and Prevention, Atlanta, GA, USA); statistical analysis was conducted by using χ^2 analysis of contingency tables. The odds ratio (OR) and 95% confidence interval were calculated. Age was described as mean ± standard deviation. The Chaim Sheba Medical Center human subjects research review board approved this study.

From January 9, 1995, through December 31, 2003, a total of 1,256,669 blood donors were screened for HTLV-1 infection in Israel. Of these, 73 HTLV-1 carriers were identified, for an overall prevalence of 5.8 infections per 100,000 donors. Average age at diagnosis was 39.4 ± 11.9 years; 48 (66%) were men (compared with 72% of all blood donors; p = 0.3125). All HTLV-1–positive donors had negative serologic results for HTLV-2, human immunodeficiency virus, hepatitis C virus, and hepatitis B surface antigen. HTLV-1 carriers originated from 20 countries (Table).

ORs for HTLV-1 carriers varied by geographic origin of donor (Figure 2). Donors from Middle Eastern and Eastern European countries were at highest risk for HTLV-1 carriage.

Conclusions

The diversity of the population in Israel, combined with systematic screening of blood donors, enabled us to examine the global epidemiology of HTLV-1 infection. One or both parents of at least 67% of Jews in Israel were born

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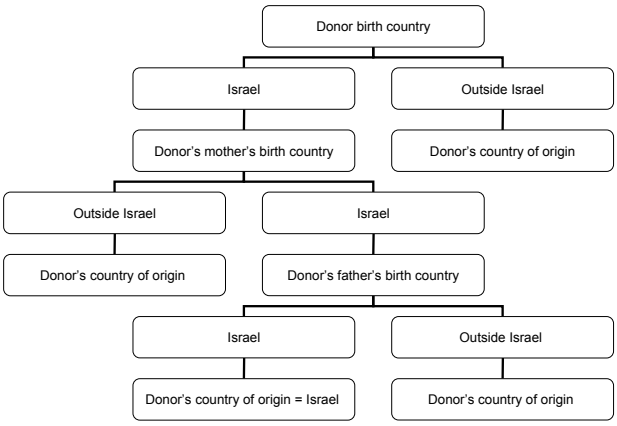

Figure 1. Algorithm for identifying the geographic origin of Israeli blood donors.

outside Israel. Thirteen percent immigrated from Asia, 16% from Africa, and 36% from Europe and the Americas (*3*). We believe that blood donors are representative of healthy persons in the general population of the country because approximately one third of the population are blood donors.

The prevalence of HTLV-1 infection among blood donors in Israel was 5.8/100,000 but varied depending on donors' geographic origins. In blood donors born in Israel, the prevalence of HTLV-1 infection was 1/100,000, comparable to and even lower than the prevalence of HTLV-1 in blood donors from northern Europe (online Technical Appendix). Unfortunately, information from many countries is either lacking or unrepresentative. We found a similar prevalence of HTLV-1 infection in Jewish immigrants from known HTLV-1 endemic countries to what is known in their native countries. For example, the reported prevalence in blood donors in Latin American countries ranged from 30/100,000 to 1,000/100,000 (online Technical Appendix); our study found a prevalence of 20/100,000.

In donors from the Middle East, we found a high prevalence of HTLV-1 infection in blood donors from Iran (50.4/100,000), Turkey (16.0/100,000), and Iraq (10.2/100,000). Of those countries, only Iran can provide information about HTLV-1 prevalence, and those data are limited to Mashhad (*4*). Our survey was performed after the initial report of HTLV-1–associated disorders in Israelis born in or originating from Mashhad (*5*). This example can highlight the role of immigrants as reflectors of the populations in their countries of origin. We found no systematic information about the epidemiology of HTLV-1 in the rest of Iran and neighboring countries. However, anecdotal reports suggest that the infection exists in Kuwait, Turkmenistan, and Georgia (*6*–*8*).

In persons from eastern Europe, we found a high prevalence of HTLV-1 carriage in blood donors originating from Romania (14.9/100,000), Russia (6.3/100,000), and the former Yugoslavia (62.9/100,000). Most of the literature does not consider eastern Europe endemic for HTLV-1 infection (*2*). Particularly in Romania, HTLV-1 prevalence is considered low, and HTLV-1 positivity was attributed mainly to immigration from other countries (*2*). In a careful review of the literature, we found only a few studies involving a small number of blood donors (online Technical Appendix). These studies support our findings and suggest that HTLV-1 is endemic in some eastern European countries. Several cases of adult T-cell leukemia have been described in patients of Romanian origin (*9*–*14*).

Our findings establish the need for rigorous screening of blood donors in suspected HTLV-1–endemic areas (Middle East and eastern Europe) and show the impact of immigrants or travelers as sentinels to the health problem in their country of origin. Our data also demonstrate high prevalence of HTLV-1 infection among those whose country of origin had been considered nonendemic for HTLV-1. Viral transmission could have occurred either from the non-Jewish local population or from other Jewish communities in which HTLV-1 is endemic. We are not aware of immigration waves of Jewish populations from Iran and Iraq to Eastern Europe and vice versa. In addition, the prevalence of HTLV-1 in blood donors from Israel is low. Therefore, it seems reasonable to conclude that our findings reflect local HTLV-1 endemicity in the country of origin.

Table. Prevalence of HTLV-1 in blood donors from different countries of origin, Israel, 1995–1998*

Country	No. HTLV-1 carriers	No. blood bank donors	No. carriers/ 100,000 donors
Iran	16	31,776	50.4
Romania	12	73,971	14.9
Iraq	7	68,857	10.2
Russian Federation	7	111,109	6.3
Turkey	4	25,054	16
Poland	3	70,172	4.3
Israel	3	294,342	1.0
Morocco	3	144,014	2.1
United States	3	49,204	6.1
Yugoslavia†	2	3,181	62.9
Uruguay	2	3,552	56.3
Argentina	2	20,898	9.6
Chile	2	2,101	95.2
Czechoslovakia†	1	11,149	9.0
Brazil	1	4,217	23.7
Niger	1	1	
Ethiopia	1	3,412	29.3
Egypt	1	21,245	4.7
Yemen	1	36,052	2.8
Libya	1	21,427	4.7

*HTLV-1, human T-lymphotropic virus type 1.
†Country no longer exists.

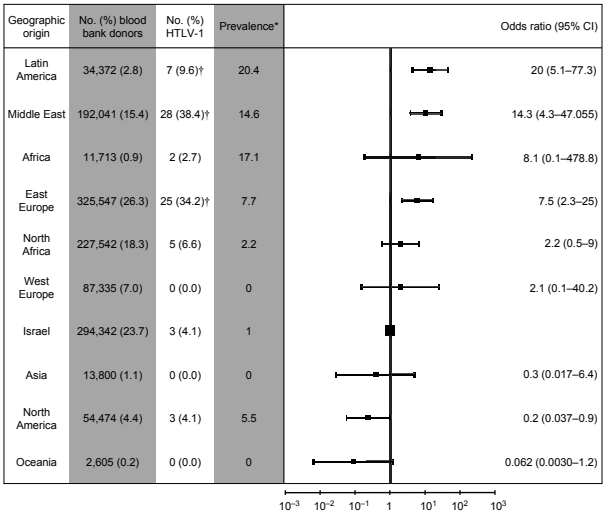

Figure 2. Relative risk for human T-cell lymphotropic virus type 1 carriage in donors from different geographic origins. Odds ratios (indicated by black boxes) are charted in logarithmic scale. Error bars indicate 95% confidence intervals (CI). *Per 100,000 population; †p<0.05.

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Recurrent Lymphocytic Meningitis Positive for Herpes Simplex Virus Type 2

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We found the prevalence of recurrent lymphocytic meningitis associated with herpes simplex virus type 2 (HSV-2) was 2.2/100,000 population in Finland during 1996–2006, higher than previous estimates. PCR was most sensitive in detecting HSV-2 DNA from cerebrospinal fluid if the sample was taken 2–5 days after symptom onset.

Recurrent lymphocytic meningitis (RLM) is a rare disease, characterized by attacks of sudden onset aseptic meningitis followed by complete recovery and unpredictable recurrences. The disease is most often caused by herpes simplex virus type 2 (HSV-2) and less frequently by other viruses, autoimmune disorders, or medication. Symptomatic episodes of RLM usually subside within 5 years, but the total number of episodes may reach 30. Patients are typically middle-aged, and women are more often affected than men (*1*–*3*). In addition to symptoms typical of meningitis, ≈50% of patients have transient hallucinations, seizures, cranial nerve palsies, or an altered level of consciousness (*4*).

The Study

This study was conducted at Helsinki University Central Hospital, Finland, which serves a population of 1.4 million. The prevalence study covered January 1996 through December 2006. This period differed from that of the patient study because the World Health Organization's coding system changed in 1996 to the International Classification of Diseases, 10th Revision. Diagnostic codes A87, B00.3 + G02.0, B01.0 + G02.0, B02.1, G02*, G03.0, G03.1 and G03.2 were used to identify study cases.

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The patient study was conducted from January 1994 through December 2003. All patients with RLM (≥2 clinical episodes, lymphocytic predominance and negative bacterial culture from cerebrospinal fluid [CSF], and HSV-2 DNA in at least 1 CSF sample) were recruited. A structured questionnaire was used to interview patients about symptoms during and after meningitis episodes. Antibodies against HSV types 1 and 2 were tested on the study entry day, which was at least 1 month after the most recent RLM episode. Sixty-two age- and sex-matched healthy participants served as controls in the laboratory analysis.

Type-specific HSV-1 and -2 immunoglobulin (Ig) G and IgM were measured by enzyme immunoassay (HerpeSelect 1&2 ELISA IgG; Focus Diagnostics, Cypress, CA, USA; and EIAgen HSV IgM; Adaltis, Bologna, Italy). The detection of HSV DNA in CSF samples was performed as described (*5*).

Statistical comparisons between groups were made by using a permutation test for titiers of antibodies against HSV-2, and Fisher exact test for HSV seropositivity. Kaplan-Meier estimate was used to illustrate information on the cumulative proportions of the second meningitis episode.

During the prevalence study, from January 1996 through December 2006, a total of 665 patients were treated at the Helsinki University Central Hospital for lymphocytic meningitis. Meningitis was recurrent in 37 patients (5.6%). Twenty-eight patients with RLM had HSV-2 DNA in CSF. In addition, 3 patients had recurring genital herpes and elevated HSV-2 serum titers. Thus, the minimum 11-year period prevalence of RLM was 2.7/100,000 population (95% confidence interval [CI] 1.9–3.7) and that of HSV-2 associated RLM 2.2/100,000 population. HSV-2 was the likely etiologic agent in 84% of all RLM cases. Six patients (16%) had no herpetic etiology. One had systemic lupus and 1 had Sjögren syndrome; in 4 patients, etiology remained unknown.

During the patient study, from January 1994 through December 2003, 86 patients had a CSF sample positive for HSV DNA. Of these patients, 23 (27%) were diagnosed with RLM; 22 case-patients (age: mean 40 years, range 25–55 years; 18 females, 4 males) were enrolled in the study.

HSV-1 seropositivity was less common in case-patients than in controls (25% vs. 52%; $p = 0.043$). All case-patients and 19% of the controls were seropositive for HSV-2 ($p = 0.003$). IgG antibody titers against HSV-2 were higher in case-patients than in seropositive controls (median 118 vs. 79; $p = 0.034$). IgM against HSV was not detected in 96% of the episodes.

The 22 case-patients had a combined 95 episodes (mean 4.3) of meningitis. The presence of HSV DNA in CSF had been analyzed during 48 episodes (Table 1). HSV-2 DNA was present in 82% of the samples taken during the first 2–5 days, and in 46% of samples obtained

Table 1. Presence of HSV type 2 DNA in CSF, leukocyte count, and timing of CSF samples in 22 patients with recurrent lymphocytic meningitis, through December 2007, Finland*

Patient no.	Age, y	No. episodes	Period (first episode–latest episode)	HSV-2 DNA in CSF	Leukocyte counts and timing of CSF samples in PCR-tested cases
1	29	7	2000–2004	NT, P, NP, NT, NP, NP, NP	**44**,† 18,‡ 4,‡ 2,‡ 1§
2	55	9	1985–2004	NT, NT, NT, NT, NT, P, P, NP, NP	**219**,§ **221**,§ 9,† 3†
3	44	4	1990–1998	NT, NT, NT, P	**310**§
4	43	4	1988–2000	NT, NT, NT, P	**790**§
5	33	4	1994–1998	NT, P, P, P	**1,410**,§ ¶,§ **73**§
6	32	4	1996–2002	P, NP, NP, NP	**164**,§ 64,† 32,† 93†
7	48	5	1979–2002	NT, NT, NT, P, NT	**196**§
8	33	4	1998–2005	NT, P, NP, NP	**360**,† 238,§ 239#
9	36	13	1990–2007	NT, NT, NT, NT, NT, NT, NT, NT, P, NP, NP, NP, NP	**195**,§ 2,† 0,† 260,§ 1‡
10	40	4	1990–1994	NT, NT, P, NT	**40**¶
11	47	2	2001–2003	P, NP	**1,006**,§ 790§
12	40	2	1989–1997	NT, P	**325**§
13	36	2	2001–2004	P, P	**592**,§ **620**§
14	38	2	1991–1996	NT, P	**92**§
15	51	3	1975–1998	NT, NT, P	**680**†
16	38	7	1992–2006	NT, P, NT, NP, NT, NT, P	**160**,† 16,‡ **106**§
17	51	3	1984–1998	NT, NT, P	**360**†
18	39	3	1989–2004	NT, NT, P	**172**§
19	45	5	1980–2004	NT, NT, NT, P,P	**350**,§ **203**§
20	25	2	2002–2004	P, NP	**1,350**,§ 430§
21	33	4	1995–2007	P,P, P, NT	**720**,§ **306**,§ ¶§
22	38	2	1990–1999	NT, P	**645**†

*HSV, herpes simplex virus; CSF, cerebrospinal fluid; NT, HSV DNA not tested; P, HSV-2 DNA present in CSF; NP, HSV-2 DNA not present in CSF.
Boldface indicates HSV-2 DNA present in the sample.
†CNF sample taken 24–48 h after first symptoms.
‡CSF sample taken within 24 h of the first symptoms.
§CSF sample taken 2–5 days after first symptoms.
¶Leukocyte count or timing of the sample not known.
#CSF sample taken >5 days after first symptoms.

24–48 hours after the first symptoms. If the sample was obtained either earlier or later, no HSV-2 DNA was detected, despite previous HSV-2 DNA-positive episodes. The median leukocyte count during the first HSV-2 PCR positive episode was 350 cells/mm^3 (range 44–1,410 cells/mm^3). In PCR negative cases, the leukocyte counts were lower.

The median patient follow-up time was 16.2 years (range 4–32 years). The number of meningitis episodes per case-patient varied from 2 to 13 (Table 1), and the number of meningitis episodes per follow-up year was 0.28 (95% CI 0.22–0.35). The time between the first and the second episode of meningitis ranged from 1 to 216 months (median 47 months; Figure).

Eight case-patients (36%) reported paresthesias and 7 (32%) had neuropathic pain during or after meningitis (Table 2). The pain typically radiated to the extremities, followed a dermatome pattern, and could last up to several years after meningitis. Arthralgias (n = 6) and urinary dysfunction (n = 5) were common.

In 56 (61%) of the 95 episodes, case-patients received antiviral medication to alleviate symptoms. Seven case-patients (32%) used daily antiviral medication to prevent new episodes; 2 experienced new episodes despite the medication.

Five case-patients (23%) experienced their first episode of meningitis after symptomatic HSV-2 genital herpes; 4 of these case-patients were HSV-1 seronegative at that time. In 4 case-patients (18%), genital herpes recurred frequently (more than 6 episodes per year). Four case-patients (18%) had a history of labial herpes; 7 (32%) had no history of herpetic infections.

Conclusions

The prevalence of HSV-2–associated RLM has been estimated to be 1/100,000 population (*6*). According to our study, the 11-year prevalence of HSV-2–associated RLM was higher, 2.2/100,000 population. Because the disease is only periodically active with long asymptomatic periods, its accurate prevalence in the population is difficult to define.

Paresthesias, neuropathic pain, arthralgias, and urinary dysfunction were common during and after meningitis. Case reports have described paresthesias associated with meningitis (*7,8*). Radiculomyelitis, urinary retention, and neuralgia associated with HSV-2 genital herpes have been reported, but rarely in connection with RLM (*9–11*).

The case-patients in our study were less often seropositive for HSV-1, and their IgG titers against HSV-2

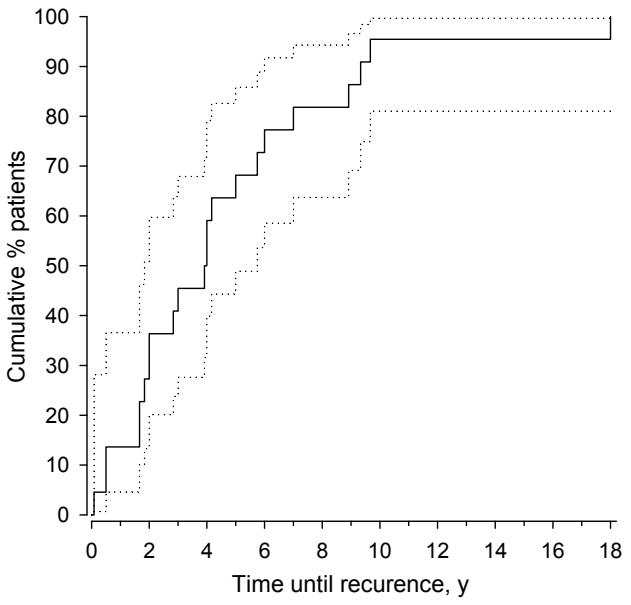

Figure. Kaplan-Meier curve showing cumulative proportions of the second recurrent lymphocytic meningitis episode (the time from the first episode to the first recurrence, years) in patients, Finland. 95% confidence intervals were obtained by bias-corrected bootstrapping.

were higher than in controls. Lately, the seroprevalence of HSV-1 has declined in developed countries, while that of HSV-2 has increased. Complications after the disease may be more frequent in HSV-2 infections without preceding HSV-1 infection (*12*).

In an earlier study, during a 1-year follow-up, recurrent disease developed in 18% of patients with HSV-2 DNA in CSF (*11*). In our study, during the 10-year follow-up, the percentage of case-patients with recurrent disease was 27% of all HSV-2 DNA-positive cases.

In RLM, all episodes are most likely caused by the same etiologic agent. However, HSV-2 DNA is not always found in CSF, despite earlier HSV-2 DNA–positive episodes. The viral load and leukocyte counts reach higher levels during the first episode of meningitis compared with recurrent cases (*13*). False-negative results may be due to a lower viral load or to earlier timing of the CSF sample in recurrent episodes (*14*). In our study, most PCR-positive samples were taken 2–5 days after the onset of acute symptoms, which is considered to be optimal timing. Clinical symptoms were alleviated in recurrent episodes, probably because of milder inflammatory changes in the CNS and lower viral load, which may result in PCR becoming less sensitive in diagnosis.

HSV-2–associated RLM is more common than previously reported. Prophylactic antiviral therapy may have decreased the incidence of recurrences but was not universally effective. In case-patients with frequent relapses, the recovery was prolonged, and residual symptoms were common.

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Table 2. Clinical symptoms in 22 patients with recurring herpes simplex virus (HSV) type 2 meningitis, during study period (January 1994–December 2003), Finland

Associated symptoms	No. patients with symptom			Maximum duration
	During meningitis episode	After meningitis episode	Total*	
Neuropathic pain	5	6	7	Years
Paresthesias	8	6	8	Months
Urinating difficulty	3	3	5	Months
Arthralgias	1	6	6	Months
Paresis of facial nerve	1	1	2	Weeks
Hallucinations	1	0	1	Days
Dysarthria	3	0	3	Days
Visual disturbance	2	0	2	Days

*Some patients experienced symptoms both during and after episodes.

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Possible Outbreak of Streptomycin-Resistant *Mycobacterium tuberculosis* Beijing in Benin

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Using geographic information system and molecular tools, we characterized a possible outbreak of tuberculosis caused by *Mycobacterium tuberculosis* Beijing strain in 17 patients in Cotonou, Benin, during July 2005–October 2006. Most patients lived or worked in the same area and frequented the same local drinking bar. The isolates were streptomycin resistant.

In a previous survey aimed at investigating the genetic biodiversity of *Mycobacterium tuberculosis* in Cotonou, Benin (*1*), we observed a higher prevalence of strains belonging to the Beijing genotype than has been reported in other studies in West and Central Africa (*2–4*). In that survey, we applied the results of spoligotyping and typing using a 12-loci mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) profile to identify the genetic lineage of the strains. In the study described here, we further investigated the identified Beijing strains by characterizing them with the more discriminatory set of 24-loci MIRU-VNTR (*5*). We also mapped the residences and workplaces of the patients by using geographic information system (GIS) technology.

The Study

From July 2005 through October 2006, a survey was conducted on 194 isolates of *M. tuberculosis* obtained from 194 patients with pulmonary tuberculosis (TB) (1 isolate per patient) (*1*). Patients were recruited from the National Hospital for Pneumology and Phtisiology in Cotonou, Benin, where most TB patients from the area are treated. Cotonou is the largest city in Benin, with a population of 655,000 in 2002 and an area of 79 km^2.

All patients gave informed consent. The study was approved by the National Tuberculosis Program Board of Benin.

Among these 194 isolates, 17 belonged to the Beijing ST1 family and exhibited the same 12-loci MIRU-VNTR pattern (223325163533). One isolate showed additional alleles at many loci. The median age of the patients infected with the *M. tuberculosis* strains belonging to the Beijing genotype (28 years) was similar to that of patients from the general survey (30 years).

Demographic data that included date of birth, age, sex, and places of residence and work were collected from each patient. Geo-coordinates of each patient's residence and workplace were obtained by using the Global Positioning System (GPS) and mapped with the ArcView 3.2 software (ESRI, Redlands, CA, USA). We also sought to map a place habitually frequented by the patients.

Blood samples for HIV testing were collected from each patient. HIV testing was performed by using an ELISA. Seropositive samples were confirmed by a discriminatory HIV1/2 test (Genie II HIV1/HIV2; Bio-Rad, Marnes-la-Coquette, France).

One sputum sample from each patient was decontaminated by using the modified Petroff method and cultured in manual Mycobacteria Growth Indicator Tube (MGIT) (*6*) and on Löwenstein-Jensen (LJ) medium. All isolates were identified as *M. tuberculosis* complex by the para-nitrobenzoic acid method and tested for drug susceptibility against rifampin, isoniazid, streptomycin, and ethambutol by using the proportion method on LJ medium at the following respective concentrations: 40 μg/mL, 0.2 μg/mL, 4 μg/mL, and 2 μg/mL (*7*,*8*).

DNA was extracted by boiling a suspension of 2 drops of MGIT-positive cultures in 300 μL of 10 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 8.0 (1× Tris-EDTA) for 5 minutes. MIRU-VNTR typing was performed at Genoscreen (Lille, France) by amplifying each of the 24 independent loci, and results were combined into digit allelic profiles (*5*).

All patients discussed here were born in Benin and had lived in the country since birth. How they became infected with the *M. tuberculosis* Beijing strain is unclear. However, because some inhabitants of Cotonou are immigrants from the Asian continent, this strain could have been brought into the country by migrant residents of the community.

In total, 6 (35%) of 17 patients were HIV-1 seropositive, and the remaining 11 patients (65%) were HIV seronegative. In contrast with the results in the initial survey,

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the *M. tuberculosis* Beijing strain was more likely to be isolated from HIV seropositive patients than from those who were HIV seronegative (21 [12%] of 173 (p = 0.024*)*.

Of the 17 *M. tuberculosis* isolates belonging to the Beijing genotype, drug susceptibility testing results were available for 16. All isolates from the 16 patients were resistant to streptomycin but susceptible to isoniazid, rifampin, and ethambutol. Of the remaining 177 isolates from the same survey, drug susceptibility testing was available for 127, of which only 13 (10.2%) were resistant to streptomycin. The *M. tuberculosis* Beijing strains in this survey were more likely to be resistant to streptomycin than were their non-Beijing counterparts (p<0.001*)*. Other researchers have suggested that *M. tuberculosis* Beijing strains may be associated with drug resistance (*9*) and that the rapid spread of Beijing strains in some settings suggests an intrinsic virulence of this family (*10*).

The combination of double alleles in several MIRU-VNTR loci of 1 isolate suggests a mixed infection (*11*). The other 16 strains showed identical profiles in the 24 MIRU-VNTR set: loci 154, 424, 577, 580, 802, 960, 1644, 1955, 2059, 2163b, 2165, 2347, 2401, 2461, 2531, 2687, 2996, 3007, 3171, 3192, 3690, 4052, 4156, and 4348 with the following profile: 244233342xx4425163353723. No amplification was achieved for loci 2163b and 2165, despite 2 rounds of PCR for all 16 isolates. This common failure to amplify loci 2163b and 2165, which are close in the *M. tuberculosis* chromosome, might be explained by a chromosomal deletion in the strain responsible for this possible outbreak. Having a chromosomal deletion supports the hypothesis that all patients were infected by the same strain. However, other possible explanations such as nucleotide polymorphisms in the sequence complementary to PCR primers could not be excluded. Many studies have reported pseudo-outbreaks caused by laboratory cross-contamination (*12*,*13*). This is not likely in the present study because almost all the specimens for primary culture were processed on different days over several months.

Mapping of patients' residences and workplaces showed that many lived or worked (or both) in the Xwlacodji area of Cotonou (Figure). To further investigate this spatial cluster and a possible link between patients, we mapped a place they habitually frequented and found that most patronized the same drinking bar (Figure). Ten of the patients either lived or worked near the bar (<300 m) or regularly visited it. Although no epidemiologic link was evident between these patients and the remaining 7 patients, most of the latter were motorcycle taxi drivers and regularly moved from place to place.

Without GPS we could not have identified the geographic cluster of these patients with Beijing strains in Xwlacodji. The residents are poor, and overcrowding creates conditions favorable for transmission of diseases from person to person and rapid spread of the resistant *M. tuberculosis* Beijing strain.

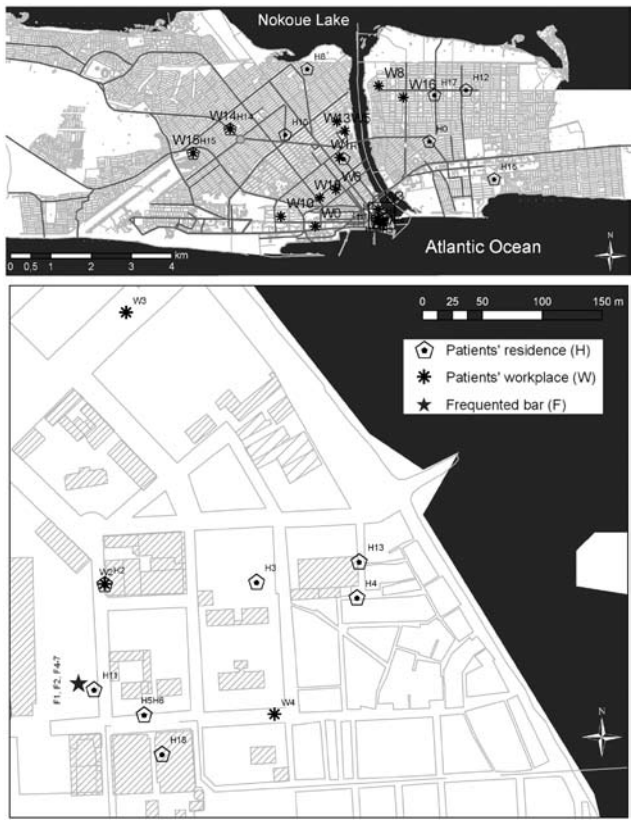

Figure. Maps showing residences and workplaces of *Mycobacterium tuberculosis* patients in Xwlacodji, Cotonou, Benin, 2005–2006.

Conclusions

We cannot completely exclude the unlikely possibility that the patients were infected by different strains with the same MIRU-VNTR pattern. However, the 17 strains' identical 24-loci MIRU-VNTR profile, with a probable deletion of the same 2 loci; the identical drug susceptibility pattern (monoresistant to streptomycin); and the fact that most patients resided in the same community as determined by GIS strongly suggest that these strains are part of an outbreak.

Generally, molecular tools are used to study TB transmission and to suggest possible outbreaks. Although molecular tools can help identify an outbreak, they cannot localize it. GIS has rarely been used in health systems, particularly in resource-poor countries such as Benin (*14*). GIS tools might be dispensable in industrialized countries where streets and houses are properly mapped and pinpointing specific addresses is generally sufficient to localize an outbreak. In many low-income settings, however, streets and houses are not properly numbered and mapped, and using GPS is necessary to gain access to accurate geo-

coordinates and localize such outbreaks. In the absence of genetic methods, differentiating cases from the same route of transmission from others is difficult. However, GIS can help TB control practitioners identify areas with aggregate cases so they can institute appropriate measures to control the disease.

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Rickettsia felis Infection in Man, France

To the Editor: In August 2008, a 64-year-old man was admitted to the Salon-de-Provence Hospital, France. He had fever (39°C) and a maculopapular rash. No eschars or adenopathy were noted. The patient had a relatively mild illness; the only abnormal laboratory values were elevated aminotransferase levels (aspartate aminotransferase 85 U/L and alanine aminotransferase 135 U/L). The man was an agricultural worker who had originated from Algeria but at this time lived in a shelter in southern France. His potential for contact with dogs in his environment was noted, but no history of flea exposure was elicted. This disease was postulated to be rickettsiosis because no other cause for his fever and rash was evident. Doxycycline was then administered, and the patient rapidly improved.

Serum testing at the Unité des Rickettsies (Marseilles, France), using a multiple-antigen immunofluorescent assay (*1*), showed the following titers: spotted fever group (SFG) (e.g., *Rickettsia felis*, *R. conorii*, *R. aeschlimannii*, *R. massiliae*) 1,024 and 512 for immunoglobulin (Ig) G and IgM, respectively, and typhus group 512 and 256 for IgG and IgM, respectively. Serum was tested by real-time PCR by using a probe that enabled screening for spotted fever and a probe specific for *R. felis*; results were negative. A Western blot with cross-adsorption (*2*) showed *R. felis* as the causative agent (Figure). At a follow-up visit 3 months later, the patient had no signs or symptoms.

Rickettsiae were first described in the cat flea (*Ctenocephalides felis*) in 1918 and tentatively named *R. ctenocephali*. However, this work was overlooked until 1990, when an ELB agent was found in *C. felis* fleas by electron microscopy (*3*); the agent was demonstrated to be a *Rickettsia*-like organism. Results of subsequent studies were controversial because of suspected contamination of cultures. The species *R. felis* was formally validated by molecular criteria in 2001, and the reference strain was isolated in 2002 (*4*). *R. felis* has been demonstrated to belong to the SFG (*5*).

R. felis is distributed worldwide (online Technical Appendix, available from www.cdc.gov/EID/content/15/7/1126-Techapp.pdf), although it has not been found in the northern, coldest regions. The vectors described include fleas, ticks, and mites; however, the only currently recognized vector is the *C. felis* flea *(6)*. The reported hosts for these vectors are mainly cats, dogs, and rodents. *R. felis* is the only SFG species that is transmitted by fleas. Studies have confirmed that *R. felis* in *C. felis* flea populations is mostly maintained by transstadial and transovarial transmission (*7*). Levels of *R. felis* infestation in *C. felis* fleas are variable, and the specific mechanisms of maintenance within each flea remain unknown. Prevalence is increased by fleas feeding on mammalian hosts infected with *R. felis*. Nevertheless, the precise relationship between the vector and the host remains unknown, and the mechanisms of rickettsial replication have not yet been examined (*7*).

We searched PubMed and found reports (case reports and seroprevalence studies) of 68 *R. felis* infections. Cases have been reported in the Americas, Asia, Tunisia, and Europe (online Technical Appendix). Such clinical cases rarely occur in warm countries, unlike the worldwide distribution of the bacteria, mentioned above. Reports of human infection with *R. felis* are rare, but the organism is frequently isolated from fleas.

We summarized the available clinical findings for 34 persons infected with *R. felis*: 32 had fever; 24, cutaneous rash (mostly maculopapular); 4, cutaneous eschar; 5, neurologic signs; 7, digestive symptoms; 3, cough without pneumonia; and 2, pneumonia. Clinical findings for *R. felis* are often confused with those found for patients with murine typhus or other febrile illnesses, and they appear to be more complex and more severe than initially thought.

R. felis infections can be diagnosed by serologic testing (*1*), molecular analysis, or a combination of each. Several molecular methods for detection of *R. felis* have focused on the presence of several genes, but real-time PCR assays are becoming increasingly useful (*8*). Serologic profiles for *R. felis* infections differ; cross-reactions with SFG rickettsiae as well as with

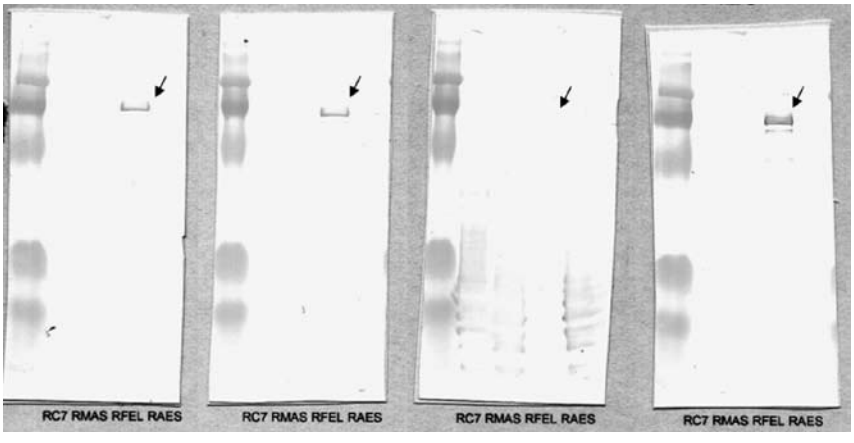

Figure. Western blot after cross-adsorption with (left to right) *Rickettsia conorii*, *R. massiliae, R. felis*, and *R. aeschlimannii*. When cross-adsorption is performed with *R. felis*, the specific antigen-corresponding line disappears, which indicates *R. felis* as the causative microorganism.

SFG rickettsiae and *R. typhi* have been observed, but a lack of cross-reactions has also been observed (*9*). It has been noted (*10*) that when cross-reactions were observed between *R. felis* and both *R. conorii* and *R. typhi*, the infection was probably related to *R. felis*. When cross-reactions were observed between *R. felis* and only *R. typhi*, the causative agent was most probably of the typhus group.

R. felis infections occur globally and are linked to the worldwide distribution of vectors, but the occurrence is relatively rare when compared with the high frequency of *R. felis* infections related to flea infestation. Human infections remain poorly characterized and are apparently underappreciated, possibly because of the lack of specific signs and symptoms. Further characterization of the interactions between *R. felis* and fleas could elucidate the particular epidemiology and pathology of flea-borne spotted fever.

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Rapid Increase of Scrub Typhus, South Korea, 2001–2006

To the Editor: Scrub typhus, or tsutsugamushi disease, is a febrile illness caused by the rickettsial bacteria *Orientia tsutsugamushi*. Scrub typhus is endemic to a geographically distinct region, the so-called tsutsugamushi triangle, which includes Japan, Taiwan, China, and South Korea (*1*,*2*). Scrub typhus is a public health issue in Asia, where 1 billion persons may be at risk for the disease (*3*). In South Korea, scrub typhus is the most common rickettsial disease, and public health authorities are concerned about its increased incidence.

Scrub typhus has been a reportable disease in South Korea since 1994. Physicians who diagnose suspected or confirmed cases must report these cases to their local health bureau and the Korea Centers for Disease Control and Prevention (KCDC) through the National Notifiable Disease Surveillance System (NNDSS). For a patient's illness to meet the case definition for scrub typhus, the clinical signs (acute febrile illness and skin eschar) must be present or there must be laboratory confirmation (4-fold rise in antibody titer, antigen detected in blood, or genetic material detected by PCR).

We analyzed NNDSS data confirmed by KCDC and classified all reported cases into 2 groups according to residential area. Cases with rural administrative address codes "eup" or "myun" were defined as rural cases, whereas cases with a city administrative address code of "dong" were defined as urban cases. All case-patients were classified by occupation as farmer or nonfarmer; all agricultural, fishery, and forest workers from rural areas were defined as farmers.

In total, 23,929 cases, including 16,199 (67.7%) serologically con-

firmed cases, were reported between 2001 and 2006, of which 35.5% were male patients and 64.5% female patients. The greatest number of cases was in the age group 50–69 years, in both male (47.2%) and females (51.7%) patients; however, there were 167 boys (2.0%) and 119 girls (0.8%) <10 years of age. The number of cases peaked in 2005, with 2,331 and 4,449 cases in male and female patients, respectively. In 2006, a total of 6,480 cases (2,364 and 4,116 in males and females patients), which is 2.5× the number reported in 2001, were reported. The autumn epidemic period was from October through November; 96.2% of all cases were reported during this period (Figure). The proportion of cases identified in farmers decreased from 2001 (44.4%) to 2006 (36.4%); the number of cases in nonfarmers reached 4,121 (63.6%) in 2006. The number and proportion of patients living in urban areas increased from 1,059 (40.2%) in 2001 to 3,230 (49.9%) in 2006. This trend was observed in both farmers and nonfarmers. The number of cases among farmers living in urban areas increased from 150 (12.8%) to 443 (18.8%), while the corresponding number of cases in nonfarmers went from 909 (62.0%) to 2,787 (67.6%). In addition, we identified different features of scrub typhus epidemicity, compared with those reported in previous studies (*4*–*7*). Many of the values reported in this study (64.5% of cases in female patients, 59.5% in nonfarmers, and 96.2% occurring in autumn) are higher than the values reported previously in Japan (*4*), Taiwan (*5*), and China (*6*). The higher incidence in female workers may be associated with conventional South Korean working behavior. Female workers typically work in a squatting position, with bare hands, and usually in dry fields, whereas male workers tend to work in a standing position, with tools, and in rice fields. Therefore, female workers are more likely to be exposed to infected mites.

Previously, farmers were considered a high-risk group, but our results imply that the same or even more attention should be given to nonfarmers. *Leptotrombidium pallidum*, a common mite in Korea, first appears in September. Its population then peaks in October and November and to a lesser degree in April and May (*7*). In autumn, especially around Chusok (Korean Thanksgiving), nonfarmers and urban residents also take part in agricultural activities, such as the chestnut harvest, mowing around graves, and assisting their farmer relatives. A sharp peak in the number of cases occurred during October–November, which is inconsistent with a previous report on vector density showing a secondary peak during April–May (*7*). This finding suggests that many cases are misreported, especially in spring. Unfortunately, there are still no reports on the comprehensiveness of the scrub typhus surveillance system in South Korea. We cannot exclude other modes of exposure such as golf, climbing, and other outdoor leisure activities. A 5-day work week was introduced in 2004, and, as a result, more leisure time has been available to urban residents. In addition, improved surveillance and diagnostic methods as well as changes in atmospheric temperature (*8*) may have contributed to the increase.

We report the rapid increase of scrub typhus and the proportion of infected persons living in urban areas in South Korea. This information will be used to establish strategies for prevention, surveillance, and management in South Korea and in other countries where scrub typhus is endemic.

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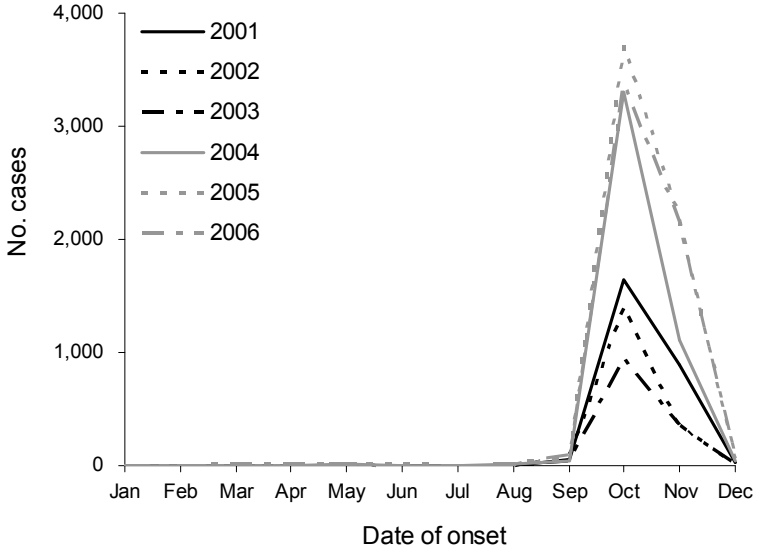

Figure. Monthly occurrence of scrub typhus cases in South Korea, 2001–2006. A color version of this figure is available online (www.cdc.gov/EID/content/15/7/1127-F.htm).

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Fatal Algaemia in Patient with Chronic Lymphocytic Leukemia

To the Editor: *Prototheca* species are achlorophyllic lower algae, ubiquitous in nature, which can cause human infections, particularly in immunocompromised patients (*1*). Human protothecosis is mostly caused by *P. wickerhamii* and *P. zopfii*. Although such infections are infrequent, they can manifest themselves clinically as cutaneous lesions, olecranon bursitis, and, even more rarely, as disseminated or systemic infections (*1*). These infections occur in severely immunocompromised patients, such as persons with AIDS, or patients undergoing extensive treatment, such as cancer treatment or organ transplantation (*1–4*). We describe a fatal case of *P. wickerhamii* algaemia in a patient with chronic lymphocytic leukemia.

In July 2007, a 79-year-old man, who had been monitored since 1993 for stage C chronic lymphocytic leukemia (*5*), was hospitalized July 13–20 for a depressive syndrome with fever, asthenia, and weight loss (3 kg over 2 months). Blood and urinary cultures on admission were sterile. The patient was hospitalized again on July 30 for fever (39°C), anorexia, and diarrhea, with ≈7 stools per day. He had lost 10 kg in 2 weeks. Blood cultures for bacteria (in BD Bactec Plus Aerobic/F and BD Bactec Lytic Anaerobic/F vials; Becton Dickinson, Le Pont de Claix, France) and fungi (BD Bactec Mycosis IC/F; Becton Dickinson) and stool cultures for bacteria were negative. Blood cultures were incubated in a Bactec 9240 instrument (Becton Dickinson). *Aspergillus fumigatus* was found in a bronchoalveolar lavage specimen, but no *Aspergillus* galactomannan antigen was detected in blood.

The patient was treated with piperacillin-tazobactam, ciprofloxacin, acyclovir, voriconazole, and loperamide. Voriconazole (400 mg/day) was used from day 17 to day 27. On day 21, *Cryptosporidium parvum* was detected on parasitologic stool examination. Symptoms persisted on day 26, with strong asthenia and deterioration of general state. At that time, the leukocyte count was 178×10^9/L with 3.56×10^9/L polymorphonuclear neutrophils and 172×10^9/L lymphocytes. Three peripheral blood samples were cultured for detection of bacteria and fungi. On day 27, septic shock developed in the patient. A blood culture showed an *Escherichia coli* strain susceptible to piperacillin-tazobactam, aminoglycosides, and quinolones. Amikacin was added to the treatment regimen. Nonetheless, the patient died on day 28.

Two blood cultures for bacteria in aerobic vials grew the day of the patient's death, but tests of blood cultures for fungus remained negative. After Gram staining, gram-positive spherical unicellular organisms were observed (Figure). After 48 hours of incubation, creamy, yeast-like colonies grew on chocolate agar (bioMérieux, Marcy l'Etoile, France), but not on Sabouraud agar containing gentamicin and chloramphenicol (Becton Dickinson). Microscopy and the API 20C AUX system (bioMérieux) identified *P. wickerhamii.*

Sequencing the 18S rDNA with the primers Pw18SF 5′-TCAAAAA GTCCCGGCTAATCTCGTGC-3′ and Pw18SR 5′-CGCTTTCGTGCCT CAATGTCAGTGTT-3′ confirmed the identification. The sequence of the amplified product was compared with sequences published in the database of the National Center for Biotechnology Information (Bethesda, MD, USA). The most likely identification, according to BLAST analysis (www.ncbi.nlm.nih.gov/blast/Blast.cgi), was *P. wickerhamii*.

In vitro susceptibility tests were performed by the Etest method (AB Biodisk, Solna, Sweden), on RPMI agar. *P. wickerhamii* was found to be

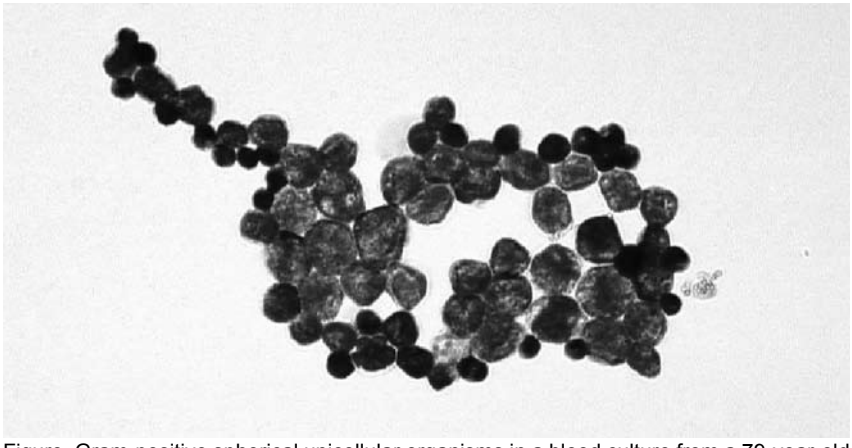

Figure. Gram-positive spherical unicellular organisms in a blood culture from a 79-year-old man with chronic lymphocytic leukemia. Magnification ×1,000. A color version of this figure is available online (www.cdc.gov/EID/content/15/7/1129-F.htm).

susceptible to amphotericin B and posaconazole, with MICs of 0.047 μg/mL and 0.012 μg/mL, respectively. By contrast, it was resistant to fluconazole (MIC>256 μg/mL), voriconazole (MIC>32 μg/mL), and caspofungin (MIC>32 μg/mL). It was also susceptible to gentamicin (MIC = 0.25 μg/mL) but resistant to amikacin (MIC>24 μg/mL).

However, the patient died before the algae were detected in the blood culture vials. In this case, antifungal treatment based on voriconazole use was empiric and ineffective. Some authors have described a successful treatment on localized protothecosis with voriconazole (*6*). Amphotericin B currently seems to be most effective agent, although the best treatment remains a matter of debate (*1*,*4*,*7*). Although in vitro susceptibility test results are not necessarily well correlated with results obtained in vivo, the low MIC of posaconazole reported here may be of interest in clinical practice (*8*). In the laboratory, use of selective yeast media, such as Sabouraud plus gentamicin, or Mycosis IC/F vials for blood culture, which contain chloramphenicol and tobramycin, may make it difficult to detect *Prototheca* spp., which are susceptible to these antimicrobial drugs.

In patients with algaemia, *Prototheca* spp. are often associated with bacteria, viruses, or yeasts which cause co-infections (*1*), as in this case, in which the alga was associated with *E. coli*. This association is probably the result of disseminated protothecosis in severely immunocompromised patients, and the alga may cross digestive or cutaneous barriers. Reasons for septic shock or death are unclear for most associations of pathogens (*2*,*4*). *Prototheca* spp. are found in various reservoirs, including the environment, animals, and food (*1*). In the case described here, the infection may have originated from a contaminated well used to obtain water for the patient's kitchen garden. However, we were unable to test this hypothesis.

Disseminated protothecosis is currently rare but, due to the algae's ubiquitous nature, increasing use of immunosuppressive therapy, and increasing incidence of hematologic malignancy, *Prototheca* spp. may emerge as opportunistic pathogens. *Prototheca* spp. should also be considered as an emerging cause of systemic infection in immunocompromised patients.

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Arcanobacterium pyogenes Sepsis in Farmer, Brazil

To the Editor: *Arcanobacterium pyogenes* is a normal inhabitant of the mucous membranes of domestic animals, such as cattle, sheep, swine, and goats (*1*). Diseases caused by this agent have been reported for persons who live in rural areas and have underlying illnesses such as cancer and diabetes (*2–4*). A recent literature review (*3*), elicited by a case of *A. pyogenes* endocarditis, found 13 unequivocal cases of human infection with this agent; many patients had a history of close contact with domestic animals. However, septicemia was not reported.

In June 2006, a 27-year-old immunocompetent man was hospitalized in Campinas (São Paulo, Brazil) for fever, cough with purulent bloody sputum, and discharge from and pain in both ears. The patient was a farmer who lived in the rural Amazon area and had extensive contact with cattle and swine. For the past 3 days he had been taking amoxicillin, 1.5 g/day, for chronic otitis media. At the time of hospital admission, his temperature was 38.9°C, respiratory rate 24 breaths/min, and blood pressure 100/70 mm Hg. He had palpable hepatosplenomegaly, but no murmur was detected in the precordium. Computed tomography (CT) scan of his chest showed multiple pulmonary nodules and alveolar infiltrates with small cavities suggestive of septic infarctions. Abdominal CT scan confirmed hepatosplenomegaly with focal lesions in the spleen. CT scan of the middle ear showed bilateral cholesteatoma and mastoiditis. No abnormalities were found during 2 transthoracic echocardiography procedures. Laboratory values were PaO_2 63.4 mm Hg, hemoglobin 11.9 g/dL, thrombocytes 109 × 10^3/mm^3, leukocytes 11.05 × 10^3/mm^3 with a left shift, serum albumin 2.6 g/dL, alanine aminotransferase 108 U/L, and total bilirubin 2.57 mg/dL. Blood and the ear secretion were submitted for culture. Cefepime was prescribed, 2 g/twice a day.

Three blood cultures grew gram-positive bacilli, initially identified as *Corynebacterium* spp., sensitive to penicillin, ampicillin, ceftriaxone, gentamicin, clindamycin, vancomycin, and resistant to erythromycin, as determined by disk-diffusion test. Ear discharge culture grew *Proteus mirabilis,* sensitive to β-lactams, cephalosporins, and aminoglycosides. Subcultures of the gram-positive bacilli on sheep blood agar grew pinpointed, grayish, β-hemolytic colonies, identified as *A. pyogenes* by use of API Coryne 2.0 kit (bioMérieux, Durham, NC, USA; code 4732761). Although susceptibility standards are not available for this organism, it was considered susceptible to penicillin and ampicillin by combining the disk-diffusion test with the MICs, as determined by the Etest (0.06 mg/L for penicillin, 0.023 mg/L for ampicillin).

Partial 16S rDNA was amplified by using primers p27f and BAC1401r and sequenced by using primers 1100r, 765fs, and 10f. Sequences were compared with those available in GenBank by using gapped BLASTN 2.0.5 software (*5*). Identification to the species level was defined as a 16S rDNA sequence identity ≥97%. Phylogenetic analysis was performed by using MEGA version 4.0 (*6*) after multiple alignments of data by ClustalX (*7*); gaps were treated as missing data. Clustering was performed by the neighbor-joining method (*8*). Bootstrap analysis was used to evaluate tree topology of the neighbor-joining data by performing 1,000 resamplings (*9*). BLASTN analysis of the 16S sequence of the isolate showed 99% identity with the 16S sequence of *A. pyogenes* (accession no. X79225). Phylogenetic analyses with MEGA grouped this isolate with *A. pyogenes* NCTC 5224 in a branch separated from other species; this grouping supported the phenotypic identification.

During the 7 days after admission, the patient's condition worsened, cefepime was withdrawn, and ampicillin 6 g/day plus gentamicin 240 mg/day were prescribed. The patient became afebrile, gradually recovered, and was discharged after 28 days of therapy.

Our patient had otitis media that progressed to sepsis, which was diagnosed by clinical, laboratory, and imaging findings. The causative agent may have been undetectable in ear discharge if it was overshadowed by a strain of *P. mirabilis,* a fastidious organism that also colonizes or co-infects this site. Endocarditis could not be ruled out because transesophageal echocardiography was not available.

A. pyogenes is usually susceptible to benzyl penicillin, ampicillin, gentamicin, and macrolides and resistant to trimethoprim/sulfamethoxazole, streptomycin, and tetracyclines (*10*). The isolate from this patient was sensitive to β-lactams, ceftriaxone, and gentamicin. However, susceptibility standards are not available because *A. pyogenes* rarely causes disease in humans. The patient had taken oral amoxicillin before admission, but his condition had not improved; subsequent addition of cefepime was also unsuccessful. The organism was probably sensitive to ampicillin, considering the low MIC and the expected serum concentration of the drug, but diffusion into the middle ear may have been poor or the local conditions caused by the cholesteatoma may have influenced the poor outcome of initial therapy. Treatment with intravenous ampicillin plus gentamicin produced full recovery.

Clinical laboratories do not routinely attempt to identify this organism. However, even in the absence of substantial concurrent illness, *A. pyogenes* must be considered as an etiologic agent of several human infections, especially septicemia, for patients with a

history of close contact with domestic animals, mainly cattle and swine.

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Reactivation of Bovine Tuberculosis in Patient Treated with Infliximab, Switzerland

To the Editor: Increased risk for reactivation of tuberculosis (TB) after treatment with tumor necrosis factor (TNF) antagonists, particularly infliximab, is well documented (*1*). We describe a case of peritoneal TB, probably resulting from reactivation of *Mycobacterium bovis* infection after infliximab treatment. In retrospect, reactivation might have been preventable had physicians been aware of the patient's history of regularly drinking fresh cow's milk from a local farm in Switzerland during 1944–45, when bovine TB was prevalent.

The patient was a 69-year-old Swiss woman who was examined for weakness, abdominal pain, increasing abdominal girth, and weight loss in April 2008. Her history included a diagnosis of Crohn disease in 1978 and treatment with glucocorticoids, cyclosporine, and mercaptopurine. In November 2007, during a recurrence of Crohn disease and while receiving treatment with azathioprine, the patient had a negative interferon gamma release assay (IGRA) result (QuantiFERON-TB in Tube test; Cellestis International, Carnegie, Victoria, Australia) and an unremarkable chest radiograph. On November 30, 2007, she was given prednisone (40 mg/d for 2 weeks, then tapered) and prescribed 3 doses of infliximab because of severe inflammation seen during colonoscopy (5 mg/kg on November 30, 2007, January 4, 2008, and February 15, 2008). She was hospitalized on April 25, 2008, at which time she had ascites; the fluid contained 1.4×10^9/L leukocytes (79.5% lymphocytes) but was negative for acid-fast bacilli on direct examination and PCR testing for *Mycobacterium tuberculosis* complex. Laparoscopy on May 2, 2008, showed extensive peritoneal inflammation. Peritoneal biopsy samples contained acid-fast bacilli and caseating granulomas; PCR for *M. tuberculosis* complex was positive. At this time, results of a repeat QuantiFERON-TB in Tube test and a tuberculin skin test (TST) were negative, but a T-SPOT.TB (Oxford Immunotec, Abingdon, UK) test was positive (6-kDa early secretory antigenic target [ESAT-6], >20 spots; 10-kDa culture filtrate protein [CFP-10], 11 spots). *M. bovis* ssp. *bovis* was grown in cultures of peritoneal biopsy samples. For culture, the MGIT 960 automated culture system (Becton Dickinson, Sparks, MD, USA) was used. The isolate was identified by use of a multiplex PCR-based, solid-phase, reverse-hybridization assay (GenoType MTBC, Hain Lifescience GmbH, Nehren, Germany), excluding *M. bovis* BCG (*2*). The patient was discharged May 30, 2008. In January 2009, she was much improved after treatment with isoniazid/rifampin/ethambutol for 3 months and moxifloxacin/rifampin for 5 months.

This case of presumed reactivation of peritoneal TB caused by *M. bovis* in a patient treated with infliximab highlights the need to be aware of local epidemiology with regard to transmissible infectious diseases.

It particularly emphasizes the value of careful history taking regarding consumption of unpasteurized dairy products in assessing risk for latent TB infection (LTBI) before starting anti-TNF treatment. Some infectious diseases experts (*3*) recommend considering LTBI treatment before prescribing anti-TNF therapy even when TST is negative or only minimally positive (<5 mm induration) for patients with epidemiologic or clinical hints of LTBI. This case indicates that this recommendation should be extended to assessment of the probability of latent *M. bovis* infection. This decision may not be easy, and referral to a TB specialist should be considered. Excluding LTBI on the basis of a negative TST result or IGRA is problematic because of the limited sensitivity of these tests, especially for immunosuppressed patients (*4*). Of note, the antigens used in IGRAs are present in *M. bovis*. Discordant TST and IGRA results are frequent, and these differences are not easily explained (*4*). However, IGRAs are not confounded by prior vaccination with *M. bovis* BCG, and the T-SPOT. TB test may have a higher sensitivity than QuantiFERON-TB and QuantiFERON-TB Gold tests (Cellestis International) (*5,6*). Our patient had a positive T-SPOT.TB result and negative QuantiFERON-TB and TST results at the time of her diagnosis of peritoneal TB.

Recently, another case of peritoneal TB due to *M. bovis* in a patient treated with infliximab was reported in Denmark. This patient's exposure presumably consisted of consumption of unpasteurized milk at a dairy (*7*). Tuberculosis cases caused by *M. bovis* are probably underreported because many laboratories determine only whether a pathogen belongs to *M. tuberculosis* complex. In the United States, genotyping of culture-confirmed *M. tuberculosis* complex has been routinely done only since 2004 (*8*).

Our patient probably acquired *M. bovis* infection by frequent consumption of unpasteurized milk in a rural area of Switzerland during the 1940s. Infection by the respiratory route is conceivable but unlikely. As the patient traveled rarely, and only in industrialized countries, more recent transmission of *M. bovis* seems unlikely. According to the requirements of section 3.2.3.10 of the World Organisation for Animal Health International Animal Health Code, Switzerland has officially been free from bovine TB since 1959. The last sporadic case occurred in 2003, although eradication has not been achieved in all neighboring European countries (*9*). Of note, in the United States and Canada, where *M. bovis* has been virtually eliminated in cattle (*10*), *M. bovis* infection continues to be diagnosed today, particularly in children of Hispanic origin and those born outside the United States. The principal infection route may be consumption of unpasteurized dairy products from Mexico (*8*). Awareness of the epidemiology of bovine TB and careful history taking regarding recent or distant (in countries where *M. bovis* infection in cattle has been eradicated) consumption of unpasteurized dairy products may prompt preventive chemotherapy before the start of anti-TNF treatment. Thus, a potentially increasing number of human TB cases due to *M. bovis* may be prevented.

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Chitinophaga terrae Bacteremia in Human

To the Editor: The genus *Chitinophaga,* first described by Sangkhobol and Skerman in 1981, belongs to the phylum Bacteroidetes (formerly the *Cytophaga-Flexibacter-Bacteroides* group), which includes filamentous, chitinolytic, gliding bacteria that transform into spherical bodies upon aging (*1*). This genus contains 10 environmental species that demonstrate similarities in 16S rDNA sequence and in phenotypic and chemotaxonomic data (menaquinone, fatty acids, hydroxy fatty acid, and polyamine) (*2*–*5*). *Chitinophaga terrae*, originally isolated from soil in South Korea, was first described in 2007 (*3*,*4*). Here we report a case of bacteremia due to *C. terrae* in a severely immunosuppressed woman.

On July 31, 2008, a 51-year-old woman was admitted to the emergency department at Nantes University Hospital in Nantes, France, because of a slowly growing left cheek mass associated with weight loss and change of general state. Physical examination showed several cutaneous infiltrated nodules, bilateral axillary adenopathies, and hepatosplenomegaly. Deteriorating renal function led to intermittent hemodialysis. Histopathology of skin and renal biopsies revealed a diffuse, high–grade, large B-cell lymphoma with cutaneous localization. Systemic CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) chemotherapy and methotrexate intrathecal chemotherapy were begun August 9. She developed bone marrow aplasia 3 days later, shortly followed by the onset of pyrexia. *Corynebacterium pseudotuberculosis* was isolated from 3 blood samples, 2 drawn using the central venous catheter (CVC) and 1 from peripheral blood. Consecutively, serotype O1 *Pseudomonas aeruginosa* strain was isolated from cultures of urine and fecal specimens. The empirical antimicrobial drug treatment started with piperacillin-tazobactam, ciprofloxacin, and teicoplanin on August 16 and was replaced by imipenem, ciprofloxacin, and teicoplanin on August 21. On August 26, the patient was admitted to the medical intensive care unit (MICU) after indications of toxic epidermal necrolysis. Blood analysis showed pancytopenia with thrombocytopenia (26×10^9/L), anemia (hemoglobin 8.7 g/dL), and profound leukopenia (0.01×10^9/L). Antimicrobial drug treatment was changed to an association of noncytotoxic drugs, i.e., aztreonam, amikacin, and teicoplanin. Despite this broad-spectrum antimicrobial therapy, 4 aerobic blood cultures (1 drawn August 29, 2 on September 2, and 1 on September 3), 2 drawn using the CVC and 2 from a peripheral site, yielded gram-negative bacilli (our laboratory reference no. NTS8639) after 2 days' incubation. The CVC was removed September 3 and sent to the laboratory for culture. The catheter tip was immersed in 2 mL of brain heart infusion agar, and semiquantitative culture was performed on the blood agar plate using 100 μL of the solution. The culture remained negative.

On September 2, treatment was changed to imipenem, trimethoprim-sulfamethoxazole, and teicoplanin. Additionally, diagnosis of invasive pulmonary aspergillosis led to changing caspofungin prophylaxis to voriconazole. Trimethoprim-sulfamethoxazole treatment was stopped September 9, and imipenem was stopped September 25, a week after bone marrow recovery. The patient was discharged from MICU on October 7.

Yellow-pigmented colonies grew on bromocresol purple lactose agar plate after 48 hours of incubation at 37°C and appeared as thin gram-negative bacilli after gram-staining was performed. The nonfermenting, nonmotile, oxidase-positive bacterium could grow at various pH values (pH 6.0, 7.3, and 8.0) and at different temperatures (30, 37, and 40°C). The semi-automatic Api 20NE gallery (bioMérieux, Marcy l'Etoile, France) identified the strain as *Sphingomonas paucimobilis*, whereas the ID-GNB card of the VITEK 2 system (bioMérieux) identified the bacterium as *Sphingobacterium thalpophilum*. The 16S rDNA amplification and sequencing were performed with universal primers 27f and 1378r as previously described (*6*). The 1366-bp sequence matched that of *C. terrae* with 100% similarity, according to BIBI (Bioinformatic Bacteria Identification, http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi) or BLAST (www.ncbi.nlm.nih.gov) analysis. Phylogenetic analysis with either the neighbor-joining or maximum-parsimony algorithm embedded the NTS8639 strain to the genus *Chitinophaga* and the species *C. terrae* (Figure). The biochemical characteristics of the bacterium corresponded to those previously described for *C. terrae* by Kim and Jung (*3*). The strain reduced nitrate to nitrite, produced N-acetyl-β-glucosamidase, phosphatase, α- and β-galactosidases, α- and β-glucosidases, and assimilated L-arabinose, L-fucose, D-glucose, maltose, D-mannose, D-melibiose, L-rhamnose, sucrose, and salicin with Api 20NE, Api ID32GN (bioMérieux), and ID-GNB biochemical galleries. Unlike *S. paucimobilis*, the strain was positive for nitrate reductase and L-rhamnose. Also, negative reaction for urease and L-fucose assimilation differentiated the strain from *S. thalpophilum*. At the species level, the bacterium grew well at 37°C, unlike *Chitinophaga arvensicola*, and showed positive oxidase reaction and nitrate reduction, unlike *Chitinophaga ginsengisegetis* (*2*,*5*).

Disk diffusion tests showed that the bacterium was multiresistant to antimicrobial drugs, including most of the β-lactams, aminoglycosides, fluoroquinolones, colistin, fosfomycin, and tigecyclin. It remained susceptible to amoxicillin-clavulanate,

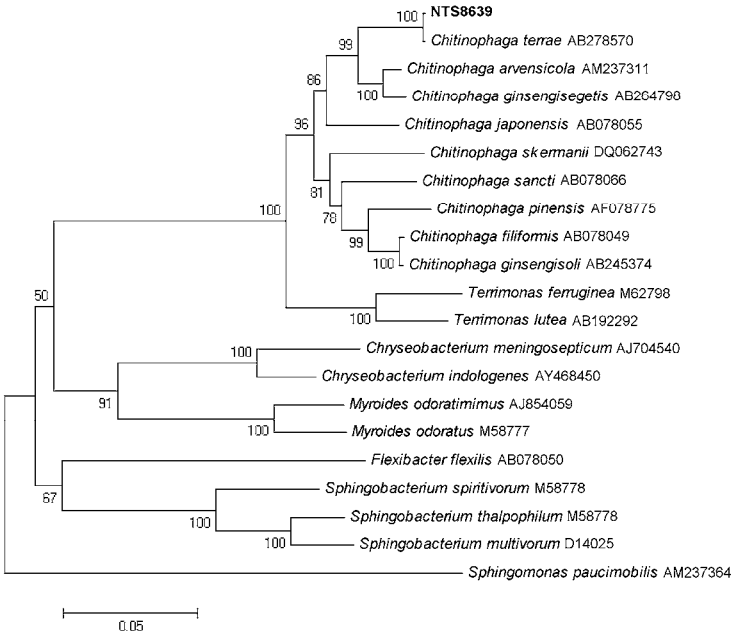

Figure. Neighbor-joining (NJ) tree showing the phylogenetic placement of strain NTS8639 (in **boldface**) among members of the *Chitinophaga terrae* species. Twenty-one 16S rRNA gene sequences selected from the GenBank database were aligned with that of strain NTS8639 by using MEGA4 (www.megasoftware.net). Accession numbers are indicated after the species name. The evolutionary history was inferred using the NJ method. The figure shows the optimal tree; the sum of the branch lengths = 1.12829943. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2–parameter method and are in the units of the number of base substitutions per site. The final dataset contains 1,304 positions. Phylogenetic analyses were conducted in MEGA4. NJ and parsimony trees were globally congruent with the distance tree and confirmed the placement of the strain NTS8639 in the *C. terrae* species. Scale bar indicates substitutions per nucleotide position.

ticarcillin-clavulanate, carbapenems, and trimethoprim-sulfamethoxazole. Interpretation of the susceptibility test was impossible before 48 hours incubation, i.e., 4 days after the first positive blood culture.

We describe in this report a case of human bacteremia due to *C. terrae*. This environmental organism behaving as an opportunistic pathogen was able to produce infection in a severely immunosuppressed woman. The source of bacteremia was not clearly established. Catheter-related bacteremia was not confirmed by culture of the CVC tip sent to the laboratory on September 3. Virulence factors contributing to the pathogenicity of *C. terrae* have not yet been well defined. The infection could have been favored by the immunosuppressive therapy, the profound leukopenia, and the extensive cutaneous detachment subsequently associated with methotrexate overdose in this patient. Unlike most susceptible environmental organisms, this bacterium probably was assisted by its multiresistance to antimicrobial drugs in producing infection. The intrinsic or acquired resistance of *C. terrae* to antimicrobial drugs has not yet been fully elucidated. The lack of commercially available biochemical gallery databases makes correct identification of this environmental organism difficult. It also underlines the usefulness of 16S rDNA sequencing for identification of unusual gram-negative bacilli isolated from immunocompromised hosts (*7*).

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Immunoglobulin G in Ebola Outbreak Survivors, Gabon

To the Editor: Three well-documented outbreaks of Ebola hemorrhagic fever occurred from 1996 through 2001 in Gabon in central Africa (*1*). All were caused by the highly pathogenic species *Zaire ebolavirus*, which is associated with an ≈80% case-fatality rate. The first outbreak hit Mayibout, a village in northeast Gabon in January and February 1996, causing 31 cases and 21 deaths. The first victims were children who helped carry and butcher a chimpanzee carcass found in the forest. The second outbreak lasted from October 1996 through March 1997 and occurred in the Booué region, about 150 km southwest of Mayibout, Gabon. The outbreak area was located along a trunk road and railroad track, and the infection spread to several villages around Booué, then to Libreville, the capital of Gabon, where 15 cases were recorded. The third outbreak occurred October 2001 through May 2002 in the Mekambo area, about 150 km from Mayibout in the east (*2*). This outbreak consisted of several independent chains of human transmission arising from infected animal carcasses, mainly chimpanzees and gorillas. It caused 65 cases and 53 deaths and coincided with major outbreaks in great apes that decimated wild populations (*3*,*4*). A total of 207 human cases were recorded during these 3 outbreaks; 149 persons died. Of the fatal and nonfatal cases 31 and 24, respectively, were confirmed by real-time reverse transcription–PCR, antigen detection, and immunoglobulin (Ig) G ELISA at Centre International de Recherches Médicales de Franceville (CIRMF) in Gabon.

Because of the lack of available samples from survivors, little is known about the duration of IgG antibody response. However, studies of 20 survivors convalescing after the 1995 Kikwit outbreak in the Democratic Republic of the Congo (DRC) showed that *Zaire ebolavirus* IgG appeared 5 to 18 days after symptom onset and persisted at least 21 months (*5*,*6*). With the exception of 2 survivors sampled 10 years after the 1976 Yambuku outbreak in DRC (*7*), no data are available on *Zaire ebolavirus* IgG persistence beyond 21 months. Low seroprevalence rates of Ebola virus or Marburg virus found in surveys of patients in outbreak areas have been attributed to seroreversion (*8*–*10*).

To investigate the persistence of *Zaire ebolavirus* IgG, we studied laboratory-confirmed survivors of the 3 outbreaks in Gabon. The study was approved by the Gabon Ministry of Health and by the traditional chief of each village, and written informed consent was obtained from each survivor. During 3 months of investigations in the different outbreak areas beginning in June 2007, we located 11, 3, and 6 survivors of the 2001 Mekambo, 1996 Booué, and 1996 Mayibout outbreaks, respectively. During home visits, the survivors underwent a brief medical consultation, malaria smears were taken, and basic medicines were provided to the villagers. We collected blood samples in EDTA tubes; plasma was separated by centrifugation in the field and stored in dry nitrogen until transfer to the CIRMF laboratory in Gabon, where it was stored at –80°C. ELISA was performed as previously described, using reagents provided by the Special Pathogens Branch, Centers for Disease Control and Prevention (Atlanta, GA, USA) (*7*). The optical density (OD) cut-off value (0.13) was calculated as the mean + 3 SD of adjusted OD values for 103 negative control serum samples obtained from Caucasian persons living in Europe.

All 20 survivors had positive test results for *Zaire ebolavirus* IgG (Table). The adjusted OD values at a dilution of 1:1,600 ranged from 0.3 to 3.4 in the 9 survivors of the 1996 outbreaks and from 0.7 to 3.5 in the 11 survivors of the 2001 outbreak. Adjusted OD values determined during the symptomatic period and/or a few days to 1 month after recovery were available for some survivors (Table). Specific IgG appeared by day 5 after

Table. Adjusted OD values in patients infected with *Zaire ebolavirus* during 3 outbreaks in Gabon, determined by testing at days 5, 10, and/or 30 after symptom onset and again in 2007 (7 or 11 years after recovery)*

Patient no.	Outbreak location and year	Adjusted OD at 1:1,600 dilution			
		Day 5	Day 10	Day 30	2007
1	Mayibout 1996	0.05	1.50	2.52	1.41
2	Mayibout 1996	0.44	0.58	1.14	0.30
3	Mayibout 1996	0.22	1.90		3.17
4	Mayibout 1996				2.09
5	Mayibout 1996			2.31	0.65
6	Mayibout 1996		1.11		3.46
7	Booué 1996		2.64		0.71
8	Booué 1996		1.56		0.49
9	Booué 1996	1.26	1.55		0.31
10	Mekambo 2001				2.60
11	Mekambo 2001		1.90		1.46
12	Mekambo 2001		2.05		0.84
13	Mekambo 2001		0.80		2.13
14	Mekambo 2001				1.84
15	Mekambo 2001				0.71
16	Mekambo 2001				2.77
17	Mekambo 2001	0.10			3.50
18	Mekambo 2001	0.46			1.33
19	Mekambo 2001	0.03			2.50
20	Mekambo 2001		2.90		0.99

*Blank cells indicate data are missing or testing was not performed. OD, optical density.

symptom onset, increased during the symptomatic period (as shown by higher titers on day 10), peaked by day 30 (2 weeks after recovery), then declined slowly over several years. *Zaire ebolavirus* IgG remained detectable, often at high levels, >11 years after the infection.

These long-lasting IgG antibody responses found in 20 survivors of 3 different *Zaire ebolavirus* outbreaks rule out the hypothesis that low Ebola virus (and Marburg virus) seroprevalence rates found in epidemic regions of Africa are due to rapid loss of specific IgG. Whether this immunity is sufficient to protect from recurrent infection remains undetermined. These findings show that IgG ELISA is suitable for epidemiologic and epizootiologic investigations of Ebola and that *Zaire ebolavirus* IgG is an excellent indicator of *Zaire ebolavirus* circulation in humans.

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Varibaculum cambriense Infections in Hong Kong, China, 2006

To the Editor: *Varibaculum cambriense* is an anaerobic, gram-positive, diphtheroid bacterium that was described by Hall et al. in 2003 (*1*). Biochemical testing, electrophoretic analysis of whole-cell proteins, and phylogenetic analysis of 16S rRNA gene sequences showed that *V. cambriense* is related to but distinct from *Actinomyces* spp. and related taxa, including the genera *Actinobaculum*, *Arcanobacterium*, and *Mobiluncus*.

Although its natural habitat remains unknown, *V. cambriense* has been isolated from intrauterine devices and human vagina and abscess specimens (*2*). Commercial systems, such as analytical profile index (API) Rapid ID 32 Strep and Coryne kits (bioMérieux, Marcy l'Etoile, France), used in differentiation of novel bacteria provide biochemical profiles useful for identification of *V. cambriense* (*1*). However, the absence of data on this organism in manufacturers' databases has hampered recognition of *V. cambriense* in routine clinical laboratories. We report 4 cases of *V. cambriense* infection and show that this bacterium is a potential pathogen in skin and soft tissue infections.

In 2006, four isolates of gram-positive curved bacilli that grew on Columbia agar with 5% horse blood under anaerobic conditions were referred by 2 regional hospitals in Hong Kong to our laboratory for identification. These isolates originated from the abscess specimens of 4 patients.

Patient 1 was a 45-year-old woman with a right ovarian chocolate cyst and endometriosis who had undergone laparotomy, right salpingo-oophorectomy, and lysis of adhesions in 2001. Since then, she had a recurrent abscess over the umbilical scar that was treated conservatively. Culture of pus from

the umbilical scar grew an unidentified gram-positive bacillus (M124). Histologic analysis of umbilical tissue showed acute suppurative inflammatory cells and microabscess formation. The patient refused follow-up and no antimicrobial drug treatment was given.

Patient 2 was a 25-year-old man who had a history of excision of multiple sebaceous cysts in the groin, and buttock pain and swelling for 4 days. He had no previous trauma and was afebrile. An abscess was incised and drained. Culture of tissue obtained grew an unidentified gram-positive bacillus (M397) and a *Peptostreptococcus* spp. The infection later subsided without antimicrobial drug treatment.

Patient 3 was a 34-year-old man with a lump in the left groin that had been present for 1 year. He was hospitalized with erythema and increased swelling of the lesion. An abscess was diagnosed, incised, and drained. Gram staining of pus showed numerous leukocytes and gram-positive bacilli. Culture yielded an unidentified gram-positive bacillus (M380). He was treated with ampicillin and cloxacillin, and the wound healed.

Patient 4 was a 55-year-old woman with an abscess on her back. She had a 3-year history of seronegative rheumatoid arthritis but was not receiving immunosuppressant therapy. The abscess was drained, and gram staining of pus showed numerous leukocytes and gram-positive cocci and bacilli. Culture yielded *Peptostreptococcus* spp. and an unidentified gram-positive bacillus (M398). The abscess healed without antimicrobial drug treatment.

The 4 unidentified gram-positive bacilli from these patients were initially characterized by using the API Rapid ID 32 Strep and Coryne kits. Doubtful identifications at various confidence levels were obtained, including *Corynebacterium diphtheriae* var. *mitis* or var. *belfanti*, *Gardnerella vaginalis*, *Streptococcus mitis*, *S. oralis*, *Gemella morbillorum*, and *Aerococcus urinae*. Sequencing of full length 16S rRNA genes suggested that the isolates were *V. cambriense*; GenBank BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) results showed *V. cambriense* type strain CCUG 44998^T sequence as the best match (identities 98%–99%) (*3*).

Biochemical reactions of the strains were similar to those of CCUG 44998^T (*1*). All 4 isolates grew poorly in air and 5% CO_2, were catalase negative, did not hydrolyse esculin or gelatin, or reduce nitrates. The 4 isolates did not ferment most of the carbo-

Table. Characteristics and test results for 4 isolates of unknown bacteria, Hong Kong, China, 2006*

Characteristic	*Varibaculum cambriense* type strain CCUG 44998^T	Isolate			
		M124	M397	M380	M398
Hospital		B	B	A	A
Patient sex/age, y		F/45	M/25	M/34	F/55
Date referred/isolated		2006 May	2006 Dec	2006 Dec	2006 Dec
API test code					
Rapid ID32 Strep		00100010100	00102010100	02122001100	00002011100
Coryne		1010321	1410321	1010721	1010321
Acid production from					
Glucose	+	+	+	+	+
Lactose	–	–	–	–	–
Maltose	V	– (+)	– (+)	+	+
Melibiose	–	–	–	–	–
Pullulan	–	–	–	–	–
Raffinose	–	–	–	–	–
Ribose	V	+	+	+	– (+)
Sucrose	V	+	+	+	+
Trehalose	V	–	–	+	–
MBDG	–	–	–	–	–
Hydrolysis of hippurate	V	+	+	–	+
Production of					
α-galactosidase	–	–	–	+	–
β-galactosidase	V	–	– (+)	–	–
α-glucosidase	+	– (+)	– (+)	– (+)	– (+)
Alkaline phosphatase	–	–	–	–	–
APPA	V	–	+	+	+
GenBank accession no.†		FJ169866	FJ169867	FJ169868	FJ169869
Identity to type strain CCUG 44998^T, %		99	98	99	99

*API, analytical profile index; V, variable; MBDG, methyl-β-D-glucopyranoside; APPA, alanyl-phenylalanyl-proline arylamidase. Test results were obtained by using the API Rapid ID32 Strep kit (bioMérieux, Marcy l'Etoile, France). Results in parentheses were obtained by using the API Coryne kit (bioMérieux).
†For 16S rRNA gene sequence.

hydrates in the 2 API kits, except for ribose, maltose, glucose, and sucrose. Discrepancies in results between the 2 test kits were seen with ribose in 1 isolate (M398) and maltose in 2 isolates (M124 and M397). One isolate (M380) did not hydrolyze hippurate but produced acid from trehalose and xylose. This isolate was also α-galactosidase positive, a result different from that of the type strain. All 4 isolates were α-glucosidase positive and 3 were alanyl-phenyl-alanyl-proline arylamidase positive. Some of the biochemical reactions for the 4 isolates, including all tests for delineating *V. cambriense* from other catalase-negative *Actinomyces* spp. (*1*), are summarized in the Table.

We report the isolation of *V. cambriense* from 4 patients with purulent skin and soft tissue infections. Our findings contribute to understanding of the clinical and pathogenic potential of this anaerobic bacterium. Gram-positive diphtheroid organisms from wound specimens are occasionally considered to be skin commensal organisms. Clinical microbiologists should be aware of this organism and the current inadequacy of commercial systems for its identification. We have shown that 16S rRNA gene sequencing is a useful alternative to gas–liquid chromatographic analyses of cell wall fatty acids or metabolic products for identification of anaerobic gram-positive bacilli.

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Outbreaks of Hemotrophic Mycoplasma Infections in China

To the Editor: Infections caused by hemotrophic mycoplasmas (formerly called eperythrozoonoses) in animals and humans have been emerging in the People's Republic of China in recent years. To date, 6 hemotrophic *Mycoplasma* spp. have been identified in rodents and mammals (*1*). *M. suis* from pigs, *M. wenyonii* from cattle, and *M. ovis* from sheep have been confirmed; the human pathogen, which is most frequently observed in China, has not been genetically identified (*2*). However, the zoonotic potential of the bacteria is evident because the disease is more prevalent in farmers and veterinary doctors, who have frequent close contact with domestic animals, than in other persons (*2*). Vertical transmission from mother to fetus has also been confirmed (*2*). In animals, especially in piglets, the disease is characterized by febrile acute anemia, jaundice, and eventual death resulting from concurrent infection with other microbes (*3–6*). Infected humans may be asymptomatic or have various clinical signs, including acute fever, anemia, and severe hemolytic jaundice, especially in infected neonates. Pregnant women and newborns were reported to be more vulnerable to the disease than others and to show more severe clinical signs after infection (*2*).

We conducted an epidemiologic investigation of hemotrophic mycoplasma infections in China by reviewing all reported cases and outbreaks for 1994–2007. Clinical cases for >6 animal species (including pigs, cows, goats, horses, foxes, chickens, and humans) were reported during the period (Table). The number of reported cases varied from year to year. Human infections were confirmed by clinical and laboratory methods (*2*). We reinvestigated blood samples of >600 pigs with previous diagnoses of mycoplasma infection accompanied by clinical signs of fever and jaundice. Slides were made and stained in Giemsa-staining solution. We used light microscopy to look for the presence of *M. suis* on the erythrocyte surface. We also used fluorescence microscopy to look for the microbes by mixing a drop of infected blood with acridine orange solution (0.1 mg/mL). The microbes bound to red blood cells were examined with a confocal microscope. Positive cases were further confirmed by PCR using primers of the small subunit RNA gene sequences. All samples were PCR positive, but PCR sensitivity is higher than sensitivity of acridine orange staining, which is higher than sensitivity of Geimsa staining.

Hemotrophic mycoplasma infection is still a neglected zoonotic disease, which poses a threat to public health and the animal industry, especially in China (*2,7*). The prevalence of the dis-

Table. Number of reported hemotrophic mycoplasma infections, China, 1994–2007*

Year	Species				
	Human	Cow	Swine	Sheep	Fox
1994	200	NR	NR	NR	NR
1995	331	132	NR	231	NR
1996	1,229	259	147	NR	NR
1997	2,262	69	1,282	126	NR
1998	740	64	127	115	NR
1999	3,861	1,460	397	2,493	954
2000	1,971	2,920	140	NR	371
2001	329	329	7,775	NR	16,697
2002	126	NR	17,068	NR	17,068
2003	880	84	600,033	1,877	31,208
2004	4	625	15,604	206	NR
2005	451	119	27,268	2,916	20
2006	4	75	15,916	536	465
2007	452	3	1,686	53	60

*NR, no record.

ease in domestic animals (e.g., pigs) and humans has reached an alarming level (Table). Human infection rates in certain areas in China have been high; for example, in Inner Mongolia, samples collected from 1,529 randomly selected persons during 1994–1996 showed that 35.3% of the local population, 57.0% of local pregnant women, and 100% of newborns of infected mothers were positive for hemotrophic mycoplasma infection (*2*). Infections in animals in China have been recognized since 1995, and the number of cases has been increasing rapidly. For example, >600,000 pigs infected with *M. suis* were reported in 2003 (Table). These infections have had a large economic impact on regions where the infection is endemic (*8*). Infections in other animals, including cows, sheep, and foxes, were also common, indicating a high prevalence of the bacteria in China. However, because of the lack of in vitro cultivation systems that assist in characterizing pathogens, progress in species identification and molecular characterization of these pathogens has been slow. Thus far, names of hemotrophic mycoplasma species have been based on the hosts from which they were identified. Due to the zoonotic nature of these pathogens, more in-depth studies on these microorganisms are needed.

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Sensitivity of Andes Hantavirus to Antiviral Effect of Human Saliva

To the Editor: Hantaviruses cause 2 severe and often fatal human diseases, hemorrhagic fever with renal syndrome (HFRS) in Eurasia and hantavirus cardiopulmonary syndrome (HCPS) in the Americas. Rodents are the natural hosts for hantaviruses that cause HFRS and HCPS, and humans are usually infected by aerosolized virus-contaminated rodent excreta (*1,2*). Except for Andes virus (ANDV), human-to-human transmission of hantaviruses does not seem to occur. ANDV clearly is transmitted directly

from human to human (*3*), but exactly how this occurs or why other pathogenic hantaviruses are not transmitted between humans is not known.

ANDV antigen has been detected in the secretory cells of the salivary glands of humans (*4*). The risk for infection with ANDV is higher in people having sex or involved in deep kissing with an infected person than in other contacts (*5*), suggesting that transmission of ANDV needs close person-to-person contact. Therefore, one can speculate that ANDV is likely to be secreted into saliva and that saliva is involved in human-to-human transmission. Hantaviruses can be transmitted through saliva between the natural hosts (*6,7*), indicating that hantaviruses can withstand the antiviral effects of saliva or can interfere with production of saliva and thereby inhibit its antiviral effect.

We recently showed that saliva from Puumala hantavirus (PUUV)–infected humans contains viral RNA (*8*). This finding suggests that PUUV, and perhaps other hantaviruses, might be secreted into human saliva. However, we found no evidence of replicating virus in the saliva samples (*8,9*); neutralizing antibodies or salivary components may have inactivated the virus. We therefore analyzed the effect of saliva on the prototype hantavirus, Hantaan virus (HTNV). Our analysis shows that although HTNV is sensitive to the overall antiviral capacity of human saliva from healthy donors, it is insensitive to the antiviral effects of certain salivary components, i.e., histatin 5, lactoferrin, lysozyme, and secretory leukocyte protease inhibitor (*9*), which are known to have antiviral effects against other viruses.

We tested the hypotheses that ANDV might be less sensitive than HTNV and PUUV to the antiviral effect of human saliva. Saliva from healthy persons with no evidence of seropositivity against hantavirus was pooled and preincubated at different concentrations with 10,000 focus-forming units of ANDV (strain Chile-9717869), HTNV (strain 76-118), or PUUV (strain Kazan E6) for 1 hr (*9*). The virus plus saliva mixtures were then titrated on Vero E6 cells. Virus without saliva was used as a control. The medium used for dilution of saliva and virus was Hank's balanced salt solution (Invitrogen, Paisley, UK) supplemented with 2% fetal calf serum, 2% HEPES, 100 U of penicillin/mL, and 100 μg of streptomycin/mL. Because of a cytopathic effect on the cells, we could not test saliva concentrations >50% (*9*). After incubation, titers in samples incubated with saliva were calculated and compared with titers from virus incubated without saliva.

The different hantaviruses clearly differed in their sensitivities to human saliva. At a low concentration (12.5% saliva), we observed a slight effect on HTNV, even though we saw no effect on ANDV and PUUV. ANDV was the only virus that resisted higher concentrations of saliva (25% and 50%), and an antiviral effect was clearly observed on HTNV and PUUV at these saliva concentrations (Figure).

Our finding that ANDV is less sensitive than HTNV and PUUV to the antiviral effect of human saliva might explain why ANDV, but not HTNV or PUUV, is transmitted between humans. Saliva might be the preferred route of transmission for ANDV between humans, as it is for the long-tailed rice rat (*Oligoryzomys longicaudatus*), the natural host for ANDV (*10*). However, transmission of ANDV between rodents, from rodents to humans, and between humans differs. Replicating hantaviruses have not been isolated from saliva of patients with HFRS or HCPS. In patients who have seroconverted, hantavirus-specific antibodies are likely to be present and might efficiently neutralize the virus, including ANDV. If this is the case, the interval might be short between excretion of the virus into the saliva and seroconversion, enabling the infected person to transmit hantavirus to other humans. Ferres et al. showed that in persons who developed HCPS after human-to-human transmission of ANDV, viremia preceded onset of disease and detection of ANDV-specific antibodies by up to 2 weeks (*5*). Sampling of saliva from healthy household contacts to ANDV-infected persons, with subsequent virus isolation attempts, might show whether human saliva is the mode of ANDV transfer during human-to-human transmission.

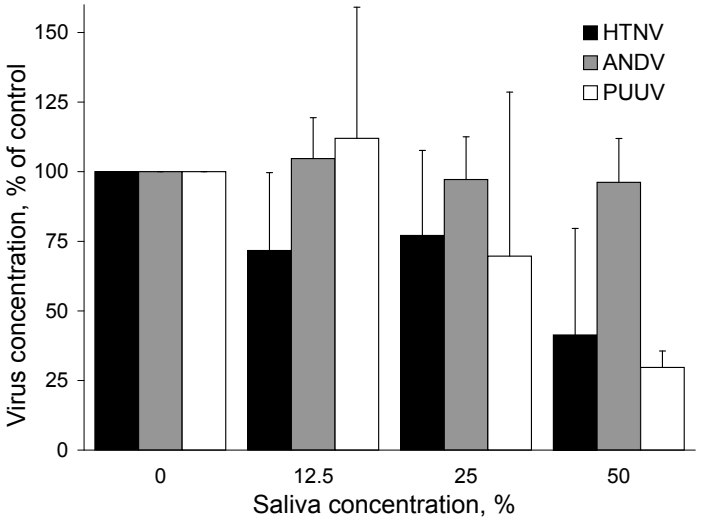

Figure. Antiviral effect of human saliva against Hantaan virus (HTNV), Andes virus (ANDV), and Puumala hantavirus (PUUV). Data represent mean + SD of 3 independent experiments.

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Spread of Cantagalo Virus to Northern Brazil

To the Editor: *Cantagalo virus* (CTGV) is a strain of *vaccinia virus* (VACV; *Poxviridae*) that was isolated from pustular lesions on dairy cattle and dairy workers in Rio de Janeiro State, Brazil, in 1999 (*1*). Subsequently, similar lesions caused by poxviruses have been reported in cattle and humans in all 4 states of the southeast region of Brazil and in Goiás State in central-western Brazil (online Technical Appendix, panel A, available from www.cdc.gov/EID/content/15/7/1142-Techapp.pdf)(*2*–*7*). Etiologic agents were VACV strains, most of which were genetically related to CTGV, such as Araçatuba and Passatempo viruses (*2*,*4*), with the exception of Guarani P1 virus, which was isolated in Minas Gerais State in 2001 and is phylogenetically close to VACV strain WR (*5*). Reisolation of Guarani P1 virus has not been reported. All VACV isolates related to CTGV share 2 molecular signatures: an 18-nt deletion in the A56R gene, which encodes viral hemagglutinin (*1*–*7*), and a 15-nt deletion in K2L gene, which encodes serine protease inhibitor-3 (*8*,*9*). Other VACV strains unrelated to CTGV were isolated from rodents in Brazil before 1999, but reisolation of these viruses has not been described (8). Although CTGV-like disease has not been reported in the northern, northeastern, and southern regions of the country, rapid interstate spread of CTGV infection is of concern. We report an episode of CTGV infection in Tocantins State, northern Brazil (online Technical Appendix, panel A).

In September 2008, teat and udder lesions were found on 15 of 356 febrile (39.5°C–40°C) cattle on a dairy farm in the municipality of Muricilândia. Small papules progressed to vesicles and pustules (online Technical Appendix, panel B), which usually healed in 3–4 weeks. New lesions subsequently appeared on previously healthy cows on the same farm, and muzzle lesions developed on suckling calves. Dairy workers reported fever and lesions on their hands and neck. The farm was quarantined for 3 weeks until the condition was diagnosed.

Four scab samples were sent for virus identification by PCR. Parts of the samples were used to infect BSC-40 cells and for DNA isolation by phenol-chloroform extraction, as described (*6*). After 48 hours, a strong cytopathic effect suggested poxvirus infection. The PCR used unambiguously differentiates CTGV-related infections from other orthopoxvirus diseases, including cowpox virus and several VACV strains (*6*). The reverse primer targets nucleotide sequences flanking the deletion signature of the hemagglutinin gene from CTGV-related viruses. Therefore, a specific annealing site for the reverse primer is produced when these external sequences are contiguous, as occurs in CTGV (*6*).

The full-length hemagglutinin gene (≈900 bp) was detected in all clinical isolates and in the control DNA samples from CTGV, VACV strain WR, and cowpox virus strain Brighton-red (online Technical Appendix, panel C). Nevertheless, when we used the primers specific for CTGV detection, only CTGV and the 4 isolates were positive, generating 714-bp fragments, which indicated CTGV as the etiologic agent. In late November, the disease was reported in 9 cattle in Santa Fé do Araguaia, a municipality 12 km west of Muricilândia. Those samples were also positive for CTGV by PCR (data not shown).

For phylogenetic inference, we used DNA from the isolate MU-07 to sequence the genes A56R (927 bp), C7L (453 bp) that encodes a host-range virulence factor, and K2L (1095 bp); primers aligned externally to the open reading frames. PCR and sequencing were performed as described elsewhere (*1*). Sequences were deposited in GenBank (accession nos. FJ545689, FJ545688, and FJ545687, respectively). Nucleotide identities in relation to CTGV sequences were 99.8% (A56R), 100% (C7L), and 100% (K2L). Both A56R and K2L genes had deletions considered to be molecular signatures for Brazilian VACV related to CTGV. Phylogenetic inference of the concatenated nucleotide dataset of 27 orthopoxviruses shows that the causative agent grouped with other Brazilian VACV related to CTGV (online Technical Appendix, panel D).

We consider the etiologic agent of the infection in Tocantins State to be a CTGV isolate, indicating spread of CTGV infection to northern Brazil. This spread could reflect interstate propagation of the virus due to movement of animals or people, which is particularly intense at the southern border with Goiás State (location of the nearest CTGV outbreak) (*3*). Nevertheless, no epidemiologic data are available to support a relationship between these episodes. The Agency for Animal Health Defense of Tocantins State has not been previously notified of clinical suspicion of poxvirus infection in dairy herds.

Another concern is spread of the virus to water buffalo, which account for a growing farming industry in Brazil, specifically in the northern states (www.ibge.gov.br/english). Infected buffalo have not yet been reported in Brazil, but the establishment of VACV strains in buffaloes has long been reported in India; economic losses have been substantial (9). Therefore, a careful survey should be conducted to evaluate dissemination of the virus to other states and species in the Amazon region.

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Acanthamoeba spp. in Urine of Critically Ill Patients

To the Editor: Free-living amebae are ubiquitous protists able to endure extreme temperature and pH in diverse environments (*1*). In past decades, interest in them increased as causes of infections, such as keratitis (*2*) and granulomatous encephalitis (*3*). *Acanthamoeba* spp. can harbor pathogenic microorganisms as endosymbionts, such as bacteria (e.g., *Legionella* spp., *Pseudomonas aeruginosa*, and *Vibrio cholera*), fungi, and mycobacteria (*4*).

The occasional observation of amebae in urine specimens in a clinical microbiology laboratory (by L.C.S.) motivated our study. To estimate the prevalence of *Acanthamoeba* spp. in urine, on March 3 and 4, 2008, we collected urine samples from indwelling urinary catheters of all critically ill patients in the intensive care units of a tertiary care 2,000-bed university hospital (Hospital das Clínicas, University of São Paulo). Medical records were evaluated for patient age, sex, underlying diseases, length of hospital stay, use of central venous catheter, mechanical ventilation, antimicrobial drug use, and duration of urinary catheterization.

Chemical urinalysis was performed by using a urine dipstick (Uriquest; Labtest Diagnostica, Lagoa Santa, Minas Gerais, Brazil), and urine leukocyte and erythrocyte counts were performed. Samples were examined microscopically and cultured for amebae. Amebae were characterized on the basis of morphologic criteria (cyst morphology and trophozoite shape and motility) (*5*). For ameba culture, 10 mL of urine was centrifuged at 2,500 rpm for 5 min. The supernatant was discarded, and the sediment (1 mL) was added to 5 mL of brain heart infusion (Oxoid, Cambridge, UK). Cultures were incubated at 25°C for 48 hr and microscopically examined. For this study, finding trophozoites on direct examination or growing *Acanthamoeba* on culture were considered a positive result for the patient.

Pipet tips (10), vacuum containers (10), plastic 15-mL tubes (10), syringes (10), glass slides (20), and tubes containing medium (10) were submitted for direct examination and culture for ameba to ensure that they were not contaminated. Urine samples were cultured for bacteria and fungi. Urine samples were submitted for bacterial and fungal direct examinations, and cultures were performed (*6*). If found, organisms were identified by morphologic, biochemical, and Gram stain characteristics.

Data from patients with and without *Acanthamoeba* spp. were compared. For dichotomous variables, we used χ^2 to calculate odds ratios and 95% confidence intervals. For continuous variables, the Mann-Whitney test was used. Results were significant at $p \leq 0.05$.

A total of 63 urine samples were evaluated; 17 (26%) were positive for *Acanthamoeba* spp. (Table). All samples of the control materials and medium tested showed negative results.

The high prevalence of *Acanthamoeba* spp. in the urine of critically ill patients is difficult to explain. Although *Acanthamoeba* spp. can cause severe infections, amebae also carry pathogenic microorganisms (*4*). Bacteria may serve as food for amebae, but other interactions exist; for example, bacteria take advantage of the protection offered by amebae, especially in the cystic form (*4*). *P. aeruginosa*, *Escherichia coli*, and *Proteus mirabilis* can infect free-living amebae (*7*). The presence of *Acanthamoeba* spp. in critically ill patients may be advantageous to potentially pathogenic bacteria in the urine, protecting them against antimicrobial drugs, disinfectants, and host immunity. In this sense *Acanthamoeba* spp. may be a reservoir for pathogenic bacterial agents in severely ill patients or, as Khan described, a "Trojan horse for bacteria" (*1*). However, we found no association between the presence of *Acanthamoeba* spp. of bacteria and fungi in the urine.

Biofilms are attractive niches for *Acanthamoeba* spp. that may provide nutritional requirements and protection against disinfectants and antimicrobial drugs (*1*). After 7 days in a patient, most urinary catheters contain a biofilm (*8*). However, we found no association between duration of catheterization and presence of *Acanthamoeba* spp. Also, *P. aeruginosa* isolates from clinical infections have shown more virulence toward *Acanthamoeba* spp. than environmental samples (*9*).

Another possible explanation is the direct pathogenic activity of *Acanthamoeba* spp. In a study to determine whether ameba-associated microorganisms are a cause of nosocomial pneumonia, in 5 of 210 cases, *Acanthamoeba* sp. was considered the only cause of infection (*10*). In our study, patients positive for *Acanthamoeba* spp. had a higher mean and median of urine leukocytes and erythrocytes, suggesting aggression by the amebae. On the other hand, even these higher counts among positive patients are considered relatively low. Cardiovascular disease, cancer, and diabetes were associated with carriage of *Acanthamoeba* spp., which may occur in more severely ill patients. As a final possibility, *Acanthamoeba* spp. in the urine could have no role at all and may even reflect contamination during catheterization.

This study has limitations. It was a small, preliminary investigation designed only to evaluate the presence of *Acanthamoeba* spp. in urine. However, our findings should lead to further studies to increase knowledge about the role of free-living amebae in nosocomial infections.

Table. Univariate analysis of variables potentially associated with *Acanthamoeba* spp. in urine samples from critically ill patients, Hospital das Clínicas, University of São Paulo, Brazil, March 2008*

Variable	Sample positive for *Acanthamoeba* spp.†	Sample negative for *Acanthamoeba* spp.‡	OR	95% CI	p value
No. (%) patients					
Male sex	10 (59)	28 (61)	0.92	0.30–2.95	0.88
Antimicrobial drug use	13 (77)	35 (76)	1.02	0.28–3.78	0.97
Use of mechanical ventilation	10 (59)	23 (70)	1.43	0.46–4.40	0.53
Presence of central venous catheter	12 (75)§	33 (72)	1.18	0.32–4.34	0.80
Urine culture positive for bacteria/fungi					
Any count	9 (53)	21 (46)	1.34	0.44–4.09	0.61
$>10^5$ CFU/mL	8 (47)	13 (28)	2.26	0.72–7.12	0.16
Underlying diseases					
Cardiovascular	15 (88)	26 (57)	5.77	1.06–41.34	0.02
Infectious diseases	10 (59)	21 (46)	1.70	0.48–6.09	0.35
Cancer	9 (53)	9 (20)	4.63	1.20–18.40	0.01
Diabetes mellitus	5 (29)	3 (7)	5.97	1.02–38.03	0.02
Renal Insufficiency	2 (12)	6 (13)	0.89	0.11–5.81	0.89
Acute abdomen	2 (12)	4 (9)	1.40	0.16–10.44	0.71
Trauma	1 (6)	11 (24)	0.20	0.01–1.74	0.11
Respiratory	1 (6)	5 (11)	0.51	0.02–5.24	0.55
Neurologic	1 (6)	4 (9)	0.66	0.03–7.19	0.71
Others	8 (47)	19 (35)	1.26	0.36–4.45	0.68
Age, y					
Mean (SD)	62.4 (13.9)	54.7 (16.2)			0.05
Median (range)	64.6 (20.5–77.5)	53.4 (17.7–80.8)			
Length of hospital stay, d					
Mean (SD)	16.3 (12.8)	13.5 (11.3)			0.45
Median (range)	11 (2–43)	9 (1–48)			
Length of ICU stay, d					
Mean (SD)	6.9 (9.0)	9.4 (10.4)			0.18
Median (range)	3.0 (1–31)	5.5 (0–47)			
Duration of urinary catheterization, d					
Mean (SD)	8.3 (9.8)	10.4 (9.4)			0.17
Median (range)	4.0 (1–33)	8.5 (1–33)			
Leukocyte count in urine per high-power field					
Mean (SD)	10.9 (17.4)	3.59 (10.53)			0.009
Median (range)	3.5 (0–60)	1.0 (0–70)			
Erythrocyte count in urine per high-power field					
Mean (SD)	35.8 (42.6)	17.7 (27.9)			0.03
Median (range)	18.5 (0–150)	4.0 (0–120)			
Urine pH					
Mean (SD)	5.4 (0.8)	5.8 (1.1)			0.18
Median (range)	5 (5–8)	5 (5–8.5)			

*OR, odds ratio; CI, confidence interval.
†n = 17 except as indicated.
‡n = 46.
§n = 16.

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Ranavirus Outbreak in North American Bullfrogs (*Rana catesbeiana*), Japan, 2008

To the Editor: Ranaviruses (family *Iridoviridae*) are emerging pathogens of farmed and wild amphibians and cause high mortality rates in these animals (*1*). These viruses are associated with massive population decreases of some species (*2*,*3*); outbreaks have been reported in the United States, Asia, Micronesia, and Europe. At the general meeting held by the International Epizootic Office in May 2008, iridoviruses of amphibians were added to the list of pathogens of wildlife that should be monitored (www.oie.int/aac/eng/Publicat/Cardsenglish/Ranavirus%20card_final.pdf, www.oie.int/eng/normes/fcode/en_chapitre_2.4.2.htm, and www.jcu.edu.au/school/phtm/PHTM/frogs/otherdiseases-viruses.htm). We report an outbreak of ranavirus disease in amphibians in Japan.

A mass die-off of wild North American bullfrog (*Rana catesbeiana*) larvae was discovered in a 1,000-m^2 pond in western Japan. The die-off lasted from September 10 through October 20, 2008, with an epidemic peak on September 20, during which several thousand carcasses were collected daily. No dead adults of *R. catesbeiana* or other amphibian species were found. Fish (families Cyprinidae and Gobiidae) in the pond were unaffected.

Clinical signs in frogs were depression; lethargy; palpebral hyperemia; abdominal edema, petechiae, and erythema on the ventral surface; skin ulcers; limb and tail necrosis; and emaciation. Pathologic changes were similar in all larvae. At necropsy, subcutaneous edema, body cavity effusions, and swollen and friable livers were observed. Histologic examination showed extensive glomerular necrosis with renal tubular hyaline droplet degeneration (online Appendix Figure, available from www.cdc.gov/EID/content/15/7/1146-appF.htm) and various degrees of hepatic cell degeneration and necrosis. Myxosporidia were not observed within any renal tubules. Electron microscopy showed cytoplasmic ranavirus-like particles within glomerular endothelial cells. These particles were icosahedral with a diameter of ≈120 nm. Bacterial colonies were observed on the skin and within multiple organs in some larvae examined. These colonies were interpreted to be opportunistic organisms and microbial cultures were not performed.

PCR with primers M153 and M154 (*4*) amplified a ranavirus-specific gene encoding major capsid protein (MCP) from 18 bullfrog specimens. DNA sequences (584 nt, which did not include primer-annealing regions) obtained from 5 PCR products randomly selected by direct-sequencing were identical. These sequences showed highest similarities with those of *R. catesbeiana* virus TW07–440 (GenBank accession no. FJ207464); only 1 nt difference was observed and this difference resulted in an amino acid substitution. Amplifications with several sets of primers (M68/M69, M70/M71, M72/M73, M84/M85, and M151/M152) (*4*) and sequencing were conducted.

We determined MCP DNA sequences of 1,472 nt that included the complete coding region (nt positions 17–1408, 1,392 nt) and proximal flanking regions. Sequences were deposited in the DNA Data Bank of Japan/GenBank/European Molecular Biology Laboratory DNA databases under the accession no. AB474588. Phylogenetic analysis showed that virus detected in this study, designated RCV-JP, showed greater similarity to TW07–440 virus than to other ranaviruses, including tadpole edema virus (*5*), frog virus 3 (*6*), and *R. catesbeiana* virus Z (*7*). Liver tissues of fish (*Gnathopogon*

spp.) that cohabitated the pond, but showed no external signs of disease, were positive for ranavirus by PCR using primers M153 and M154. Further sequence analyses are ongoing, and additional investigations of other amphibians and fishes are needed.

Live freshwater fish from several countries have been imported into Japan. However, large amounts (≤1,300 tons in 2007) of live aquaculture products, including eels and other fishes, have been imported from Taiwan into Japan (www.customs.go.jp/tariff/2007_4/data/03.htm). Given that viruses that originate in Japan and Taiwan are similar, the ranavirus we detected was likely imported into Japan in an infected aquatic organism. However, an epidemiologic survey will be necessary to determine the source of the ranavirus in the pond studied. Likewise, this virus may be endemic to Japan, and a survey of native and foreign free-ranging amphibians should be conducted. Molecular analysis of ranaviruses detected in these surveys will be necessary to differentiate endemic viruses from introduced viruses.

Japan is located at middle latitudes and has a temperate climate. This country has long been geologically isolated from Asia. This isolation has resulted in the development of many diverse species of amphibians in Japan; 23 species of the order Caudata and 35 species of the order Anura. Of these species, 49 (84%) are native and 36 (62%) are listed by the Ministry of the Environment as threatened species (*8*). *R. catesbeiana* frogs were introduced into Japan in 1918 as a food animal, and raising them by aquaculture was widely attempted. Although they are no longer cultured, feral populations have become established throughout Japan (*9*). Ranavirus in *R. catesbeiana* frogs represents a serious threat to amphibians throughout Japan.

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Maternal Antibody Transfer in Yellow-legged Gulls

To the Editor: Avian influenza viruses (AIVs) are emerging pathogens of concern because they can cause deaths in birds and humans (*1*). Although wild birds likely contribute to AIV emergence because they are the natural reservoir for all known influenza virus subtypes (*1*), our understanding of AIV transmission and immunology in natural avian populations is incomplete (*2*). In this context, the transfer of maternal antibodies is a tool that should be used more often in immunologic analysis. Because antibodies in eggs and hatchlings can reflect the mother's past exposure to pathogens (*3*,*4*) and both life stages are more easily sampled than adults, quantifying antibodies found in avian young could help clarify AIV epidemiology.

We determined whether eggs of yellow-legged gulls (*Larus michahellis*) contained antibodies against AIVs. Yellow-legged gulls can host AIVs (C. Lebarbenchon, unpub. data), are abundant, and nest in large, dense colonies in coastal areas. In April 2008, we collected 466 eggs from 2 yellow-legged gull colonies located on the Mediterranean coast: 252 eggs from Gruissan (43.1099°N, 3.1071°E; 350 breeding pairs over 1.5 hectares), and 212 from Villeneuve-lès-Maguelone (VLM; 43.4895°N, 3.8520°E; 400 pairs over 1 hectare). Villeneuve nests formed 2 spatially clustered

subcolonies: VLM1 (129 eggs) and VLM2 (83 eggs). We also collected global positioning system coordinates for VLM1 nests.

Egg yolks were isolated and frozen at –20°C until analysis. Antibodies were obtained by using chloroform extraction (*4*). The yolk was diluted 1:1 in phosphate-buffered saline and homogenized. An equal volume of chloroform was added, the solution was centrifuged (6,000 × *g* for 15 min), and the supernatant was used in the analyses.

Extracts were tested for antibodies against the AIV nucleoprotein by using a commercial competitive ELISA (IDEXX/Institut Pourquier, Montpellier, France). The assay has been validated by using seagull serum (IDEXX, pers. comm.) and chicken egg yolk (*5*). A subset of samples was tested by using a second commercial competitive ELISA (IDVet, Montpellier, France). Optical density values obtained in the 2 assays were significantly correlated ($r = 0.90$, $df = 39$, $p<0.001$), and serostatus was consistent across assays.

Overall antibody prevalence was 14% (65/466), indicating exposure to influenza A viruses in these colonies. As expected, antibody prevalence in gulls is higher than the viral prevalence previously estimated by reverse transcription–PCR or virus isolation on fecal samples, i.e., the methods typically used by avian influenza surveillance networks. A spring and summer survey performed on feces from gulls of the Camargue region (east of our colonies) showed that only 0.9% of gulls (2 infected of 228 sampled) were excreting AIVs (C. Lebarbenchon, unpub. data).

Egg antibody prevalence did not differ significantly between colonies. The antibody prevalence of 13.5% found at Gruissan (34 of 252 eggs) was comparable with the 14.5% found at Villeneuve (31 of 214 eggs) (generalized linear model with binomial distribution, $z = 0.4$, $p = 0.8$). The subcolonies of Villeneuve also did not differ: 18/129 (14%) in VLM1 compared with 12/83 (14.5%) in VLM2 ($z = 0.02$, $p = 0.9$). There was no evidence of spatial autocorrelation in the distribution of antibody in eggs by using the Moran I spatial statistic. This similarity of antibody prevalence across and within colonies suggests that exposure is dictated by regional rather than local conditions, a hypothesis that should be tested by sampling across a broader range of nest densities and over time.

Our study presents evidence for the presence of antibodies against AIVs in wild bird eggs, and the findings have important practical implications. The difference in prevalence estimated from virus isolation (0.9%) and antibody detection (14%), although expected, highlights the complementary nature of the 2 approaches. Most surveys estimate current infection by virus isolation, which provides information about disease risk in addition to phylogeographic tracking of strains. In contrast, information on antibody prevalence, which shows past and present population exposure and risk, has largely been ignored with few exceptions (e.g., *6,7*). Future work could benefit from using both approaches in tandem with modeling to develop an understanding of avian influenza ecology in nature.

Our results also show the generalizable potential of maternal antibody transfer for tracking pathogen exposure in wild birds, notably in the case of recognized emerging zoonoses. Because eggs and hatchlings are proxies of past and present adult pathogen exposure (*3*,*4*), the difficult and sometimes disruptive sampling of adults can be circumvented by the rapid and cost-efficient sampling of their young, which will facilitate monitoring efforts. Due to the high intranest correlation in egg antibodies (*4*), only partial sampling of clutches (e.g., 1 of 3 eggs) is necessary to track pathogen presence and prevalence through space and time. The sampling effects could further be minimized by taking blood samples from young nestlings of a standardized age. Finally, such samples provide abundant material for the simultaneous surveillance of other emerging pathogens of interest, such as *Campylobacter* spp. (*8*) and West Nile virus (*9*).

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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.

Prevalence of Human Bocavirus in Human Tonsils and Adenoids

To the Editor: Recently, Longtin et al. (*1*) reported a high rate (43%) of human bocavirus (HBoV) infection in a group of children chosen to serve as controls in a study of HBoV prevalence among hospitalized children and adults. In contrast, previous reports had found low HBoV prevalence rates of 0%–1% in control groups (*2,3*). Attempting to explain this surprising difference in rates, Lu et al. (*4*) suggested that selection of control patients may be related to the difference in rates. The control group used in the Longtin study was primarily (71%) made up of children undergoing elective surgery; previous studies had selected control groups from other sources, including well children on routine visits and outpatients with nonrespiratory symptoms. Because the Longtin study surgeries were mainly tonsillectomies, adenoidectomies, and myringotomies, Lu et al. examined the possibility that tonsillar tissues, which include the adenoids, are sites of persistent HBoV infection. When these researchers extracted DNA from tonsillar lymphocytes obtained from pediatric patients who had undergone tonsillectomies or adenoidectomies, they detected HBoV DNA in 32% of the samples (*4*). These findings strongly suggest a connection between HBoV and tonsillar tissue. Therefore, we tested a number of tonsillar samples for the presence of HBoV DNA.

Sample acquisition was approved by the Nationwide Children's Hospital Institutional Review Board. Tonsils and adenoids were obtained from 91 patients who underwent elective surgery at Nationwide Children's Hospital from June through September 2004. Patients' ages ranged from 1 to 16 years (median 5.9 years; age was unknown for 4 patients).

Samples consisted of surgically removed tonsil or adenoid tissues. DNA was extracted and its concentration was determined as previously described (*5*). Two primer sets were used for HBoV detection by using real-time PCR with SYBR Green detection and melting-point determination. We designed primers 3097F (5′-GTC-CAA-TTA-CAT-GAT-CAC-GCC-TAC-TC) and 3420R (5′-TGC-GTC-CAC-AGT-ATC-AGG-TTG-TTG) that targeted the viral protein 1/2 (VP1/VP2) region of HBoV. The nonstructural protein 1 (NP1) region was targeted by using primers 188F and 542R from Allander et al. (*6*) Each 20-μL reaction contained SYBR Green JumpStart Taq ReadyMix (Sigma, St. Louis, MO, USA), 4 mmol/L $MgCl_2$, 250 nmol/L primers, double-distilled H_2O, and 2 μL of DNA (50–200 ng) cycled on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA, USA) instrument at 94°C for 2 min, followed by 40 cycles of 94°C for 20 s, 60°C (NP1 primers) or 68°C (VP1/VP2 primers) for 20 s, and 72°C for 14 s. Amplification and melting curves were analyzed with 7900HT version 2.2.1 software (Applied Biosystems); positive samples were verified by sequence analysis. Sequenced VP- and NP1-generated amplicons were 99%–100% identical to HBoV strain ST1 (*6*). The detection sensitivities of the VP and NP1 assays, determined by using a plasmid construct containing the full HBoV genome, were 1–5 and 5–10 gene copies/reaction, respectively.

Our testing identified HBoV DNA in 5 (5.5%) of the 91 children who underwent elective tonsillectomy/adenoidectomy. Ages ranged from 1.9 to 4.6 years (median 3.4 years). The reason for the much lower HBoV prevalence in this group of children, compared with prevalences found in studies by Longtin et al. and Lu et al., is unclear. Lu et al. (*4*) reported a much higher HBoV rate of lymphocytes from adenoids (56%) than from tonsils (16%). Although we did not know

the exact tissue type of each sample, only that tonsils, adenoids, or both combined could be present, the 5.5% rate we found was about one third the rate found in tonsil lymphocytes and about one tenth the rate found in adenoid lymphocytes.

A seasonal effect may contribute to the large discrepancies found in HBoV prevalences. Apparently, viruses can persist in tonsillar tissue well after the symptomatic phase of illness. In children with no signs of acute respiratory infection, Drago et al. (*7*) reported that 45.5% of samples contained viral nucleic acid. Depending on the duration of persistence, asymptomatic children, sampled shortly after the season of the virus in question, would be more likely to have detectable virus in their tonsillar tissue. The Longtin et al. study samples were collected from December through April; our study samples were collected from June through September. If HBoV is seasonal, as has been suggested (*3*), it may have been circulating in the target population before samples were taken and persisted only in tonsillar tissues. Thus, if tonsillar tissue from asymptomatic children was obtained within the persistence period after the HBoV season, samples would be HBoV positive; those obtained shortly after the persistence period would have a much lower rate.

Differences in patient age in the 3 studies may also have contributed to the different rates observed. The Longtin et al. group was substantially younger (median age 23 months) than the Lu et al. group (median age 5 years) or our group (median age 5.9 years). Preliminary seroepidemiology reports indicate the presence of HBoV antibodies in >50% of children 2–3 years of age (*8,9*).

The detection of HBoV in the tonsillar tissues we tested showed a higher rate of infection than would be expected in an asymptomatic population. However, the rate was far lower than that previously reported for tonsillar tissues (*1,4*).

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Bartonella rochalimae and Other *Bartonella* spp. in Fleas, Chile

To the Editor: Fleas are involved in the natural cycle of different *Bartonella* spp. Among the 20 currently recognized *Bartonella* spp., 13 species or subspecies have been implicated in human disease. Recently, *B. rochalimae* was identified in a patient who had received numerous insect bites and subsequently had bacteremia, fever, and splenomegaly after visiting Peru (*1*). A recent study in Taiwan suggested that rodents could be a reservoir for *B. rochalimae* (*2*), but the vector or other mechanism of infection remains unknown. We amplified *B. rochalimae*, *B. clarridgeiae*, and *B. henselae* from fleas (*Pulex irritans* and *Ctenocephalides felis*) collected in Chile and dis-

cuss the role of these fleas as possible vectors of infection.

From 2005 through 2008, we collected 82 fleas from cats and dogs in pounds in Chile: 34 *P. irritans*, 37 *C. felis,* and 11 *C. canis*. Fleas were kept in 70% ethanol and sent to the Special Pathogens Laboratory of the Área de Enfermedades Infecciosas of the Hospital San Pedro, La Rioja, Spain, to be examined for *Bartonella* spp. Fleas were then rinsed in distilled water and dried on sterile filter paper under a laminar-flow hood. Each flea was crushed with a sterile pestle, and DNA was extracted by lysis with 0.7 M ammonium hydroxide. PCR was used to detect *Bartonella* DNA (according to the defining criteria for *Bartonella* spp.); primers targeted the RNA polymerase β-subunit–encoding gene (*rpoB*) and the citrate synthase gene (*gltA*) (*3*–*5*). PCR primers for a fragment of the 16/23S rRNA intergenic region and the heat-shock protein-encoding gene (*groEL*) were also used (*6*,*7*). Positive controls (*B. henselae* strain Marseille, kindly supplied by Unité des Rickettsies, Faculté de Médecine, Université de la Méditerranée, Marseille, France) and negative controls (sterile water instead of template DNA) were used. PCR products were purified, and both strands of each amplicon were subjected to sequence analysis. Nucleotide sequence homologies were searched by using BLAST (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

When *rpoB* primers were used, *Bartonella* spp. were found in 4 *C. felis* (4.8%) fleas from cats and in 4 *P. irritans* (4.8%) fleas from dogs. The same 8 samples were positive when primers for *gltA* gene and 16/23S rRNA intergenic region were used. Unfortunately, none of the 82 specimens were positive when PCR primers targeting the *groEL* gene were used. In all experiments, negative controls remained negative.

Sequencing of the 825-bp *rpoB* fragments from the 4 *C. felis* fleas indicated that they were most closely aligned with the gene sequences of *B. clarridgeiae* (n = 2, >99% similarity) and *B. henselae* (n = 2, 100% similarity). Using *gltA* (380 bp), we found 100% similarity with *B. clarridgeiae* and *B. henselae*. Accordingly, the 16/23S rRNA amplicons from these specimens exhibited 100% similarity with the corresponding sequences of *B. clarridgeiae* (154 bp) *and B. henselae* (172 bp).

Amplicons for the *rpoB* fragment gene obtained from 4 *P. irritans* fleas showed highest similarity (97.2%–99.5%) with *rpoB* of *B. rochalimae.* Three were identical, and we deposited the consensus sequence in GenBank in 2006 under the name "uncultured *Bartonella* sp." and accession no. DQ858956. The sequence differed from those described for all known *Bartonella* spp. and phylogenetically was most closely related to *B. clarridgeiae* (*8*). The sequence of the protein encoded by *rpoB* in these 3 specimens (protein_id ABH09235) had 3 aa changes (121I→V, 233K→I, and 274N→E) with respect to the deduced sequence of the RpoB protein for *B. rochalimae*. The importance of these changes remains unknown. The remaining nucleotide sequence was recently submitted to GenBank under accession no. FJ147196, designated *B. rochalimae* because isolation of this new *Bartonella* spp. was reported in 2007 (*1*). These 4 specimens also yielded positive PCR products for *gltA* (380 bp) and 16/23S rRNA (≈175 bp). Subsequent nucleotide sequence analysis showed 100% homology with the corresponding partial nucleotide sequences from *B. rochalimae*.

In 2002, Parola et al. (*9*) amplified *Bartonella* DNA by using PCR with *Pulex* spp. fleas collected from persons in Peru and suggested the existence of a new *Bartonella* sp. The nucleotide sequence of the 16S-23S ribosomal RNA intergenic spacer obtained from 1 genotype (clone F17688) was nearly identical to the corresponding sequence of *B. rochalimae*. This finding suggests that *Pulex* spp. fleas could be vectors.

Cat scratch disease has been reported in Chile, and *B. henselae* has been found in cats in Chile (*10*). Thus, our finding of *B. henselae* and *B. clarridgeiae* in *C. felis* fleas from Chile confirms the risk for exposure of humans in contact with cat fleas. Furthermore, our finding of *B. rochalimae* in *P. irritans* fleas from dogs in Chile supports the possibility that *P. irritans* fleas could be vectors for *B. rochalimae*. These findings are of public health importance because they identify possible vectors of these human pathogens.

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Emerging Infections in Asia

Yichen Lu, Myron Essex, Bryan Roberts, editors

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Emerging Infections in Asia comprises a selection of scientific and historical review chapters on a variety of infectious diseases and includes contributors from diverse locations ranging from Saudi Arabia to Australia. The book arose from the editors' experience with the emerging infections described and from their professional associations with the other contributors. The book is divided into 3 disease-specific sections that focus on avian influenza, severe acute respiratory syndrome (SARS), and HIV/AIDS, respectively; a fourth section contains short reviews of other infections. Because of the relatively small size of the book for such a broad topic (250 pages), each section covers only a few specific topics for each selected disease.

The book contains some well-written reviews and valuable narrative histories that are important to document (e.g., the 2003 SARS outbreaks in Singapore and Taiwan). The chapters on SARS in animals and emerging paramyxoviruses are particularly interesting, and the book includes topics (e.g., *Escherichia coli* and *Staphylococcus aureus*) that are often overshadowed by more glamorous emerging infections. However, it is disappointing in that the first 2 chapters contradict one another on the occurrence of person-to-person transmission of influenza virus A (H5N1), and the chapter on SARS in China could have been improved with more rigorous editing. Most chapters appear to have been written around 2006, and although they provide good snapshots of the state of knowledge at that time, readers will need to look elsewhere for more recent developments.

Emerging Infections in Asia also misses an opportunity to pull together the diverse topics and experiences discussed to provide new insights into the emergence of infectious diseases and into responses to the constantly shifting challenge of emerging infections. Recognizing this drawback, the editors say in their preface, "We hope that our book can help readers make their own conclusions and ask more questions." Approached in this context, the book provides an account of the diversity and challenges of emerging infectious diseases in Asia and some informative historical reviews that will be of interest to students exploring this fascinating topic.

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War and Disease: Biomedical Research on Malaria in the Twentieth Century

Leo B. Slater

Rutgers University Press, Piscataway, NJ, USA, 2009
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Pages: 272, Price US $45.95

War and Disease is a fascinating historical account of the discovery of drugs effective against malaria, one of the great scourges of humankind. The author, Leo B. Slater, makes good use of his expertise as a historian of biomedical science and technology. He provides a meticulous reconstruction of the manner in which the scientific community, in the midst of World War II, established an antimalarial program, which was to biomedical research what the Manhattan Project was to the physical sciences. At a time when industrialized nations are involved in the effort to find solutions to the ongoing global health catastrophe that malaria is today, this volume is a timely and valuable contribution.

Malaria's effects have long been at the center of colonial expansion and war. The disease became a focus of research in the late nineteenth century. France and Great Britain had expanded their colonies into areas of the world where malaria was the most severe and debilitating of the parasitic tropical diseases—a factor that limited the colonial governments' exploitation of natural resources. Malariologic research thrived in these countries and in Italy, where the disease was not a colonial problem but an endemic scourge and overwhelming obstacle to development, just as it is in many developing countries today. From 1880 through 1898 research carried out by such prominent scientists as Charles-Louis-Alphonse Laveran, Ronald Ross, Angelo Celli, Ettore Marchiafava, and Giovanni Battista Grassi led to the discovery of the malaria transmission cycle**.** The neurologist, Camillo Golgi, who shared the 1906 Nobel Prize for his work on the structure of the nervous system, studied the reproductive cycle of the parasite (*Plasmodium* spp.) and elucidated the synchronicity between the symptoms of recurrent chills and fever and the rupture and release of merozoites into the blood. These findings offered an explanation for the effectiveness of treatment with quinine, which had been used empirically as a generic febrifuge since the 17th century. The alkaloid remained the only effective

option for treating malaria until the 1930s.

During World War II the disease reached epidemic proportions among American troops fighting the Japanese in the South Pacific. Quinine was the main line of defense, but the supply of quinine was cut off by Japanese military conquest. The development of new antimalarial drugs became a major subject of research. Three chapters of the book—Preparing for War, Preparation and Coordination, Trust and Transition—focus on the comprehensive research program, which integrated exceptional technical and scientific expertise into a massive organizational and cooperative effort. Funded by the United States Office of Scientific Research and Development, this program screened ≈14,000 compounds for antimalarial efficacy. Clinically approved Atabrine (quinacrine) became the drug of choice in 1943, and, shortly after the war, chloroquine was identified, which has had an enduring influence on antimalarial chemotherapy. The wartime effort, Slater argues, was essential to the development of the US National Institutes of Health. Making a critical point, however, he suggests that although the wartime antimalarial program is an excellent model for future large-scale biomedical research projects, it is "a potential example of how not to pursue public health research for impoverished civilians" (p. 2). The transfer of research results into health benefits for the most vulnerable persons in malarial lands was not on the agenda. The widespread usefulness of a drug such as chloroquine was only a side effect.

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Pieter Bruegel the Elder (c. 1525–1569) The Fall of the Rebel Angels (1562) Oil on panel (117 cm × 162 cm) Musées Royaux des Beaux-Arts de Belgique, Brussels. Photo: Lefevre

Awake, Arise, or Be for Ever Fall'n

—John Milton, Paradise Lost

Polyxeni Potter

"Pieter Bruegel was the most perfect of his century," said Flemish cartographer and geographer Abraham Ortelius eulogizing his friend, who "was taken from us while still in his full manhood." Both men observed and delineated new and original angles of reality, Ortelius in his great atlas (Theater of the World), Bruegel in his paintings, which described, in astonishingly modern terms, the lives of ordinary people. "When asked which of his predecessors he followed, the painter Eupompos is said to have declared that he followed nature herself, not an artist. This agrees with our Bruegel …. Indeed, I would not call him the best of painters, but rather the very nature of painters. So I think that he is worthy of being followed by all," noted Ortelius, not alone in his praise.

"Nature was wonderfully felicitous in her choice when, in an obscure village in Brabant, she selected the gifted and witty Pieter Breughel to paint her and her peasants and to contribute to the everlasting fame of painting in the Netherlands," wrote Karel van Mander in his Book of Painters in 1604. The obscure village was Breughel, a name the painter took for himself, though he later dropped the "h" in its spelling. Not much is known about him outside van Mander's account. "He learned his craft from Pieter Koecke van Aelst, whose daughter he later married." He settled in Antwerp and joined the guild of painters. He did much work for Hans Franckert, a merchant, who joined him often in his excursions among the peasants to know and paint them. He traveled to Rome but lived most of his 44 years in Antwerp and Brussels.

He was a "quiet and able man who did not talk much but was jovial in company, and he loved to frighten people, often his own pupils, with all kinds of ghostly sounds and pranks that he played." His penchant for levity carried into his work. "He practiced a good deal in the manner of Jeroon [Hieronymus] van den Bosch and made many similar weird scenes and drolleries," van Mander wrote, "Indeed, there are very few works from his hand that the beholder can look at seriously, without laughing. However stiff, serious, and morose one may be, one cannot help laughing or smiling."

Mirth was welcome in Bruegel's times, much as today. The Netherlands, then a kingdom under Spanish rule, was torn by religious fanaticism, and many were killed for heresy. Some of Bruegel's works are allegories of the struggles between religious factions, but "it would be very hard to enumerate everything Bruegel did—fantasies, representations of hell, peasant scenes, and many other things …. He painted a picture in which Lent and Carnival are fighting; another, where all kinds of remedies are used against death; and one with all kinds of children at games; and innumerable other little, clever things." His paintings were popular in his lifetime. They were collected by the Habsburgs and brought high prices; 49 works survive. Yet his reputation among the critics suffered from his overstated connection with Bosch and from his reluctance to follow the idealized styles prescribed by the Italian Renaissance.

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Bruegel painted The Fall of the Rebel Angels, on this month's cover, while he lived in Antwerp. This work, one of very few in the tradition of Bosch, shows Bruegel's inclination toward the monumental, an aspect of the Italian Renaissance he did adopt. This, like many of his other works, shows a panoramic scene entirely filled with figures. The simplicity of form that characterizes these figures is what distinguishes Bruegel from other Flemish artists of his generation. The flat shapes, clean contours, and expressive faces devoid of extraneous detail are readily recognizable.

The fall of the rebel angels was described in the Book of Revelation as conflict between Archangel Michael and the forces of good against "that ancient serpent" and his fallen angels. The conflict, a favored theme in art from the Middle Ages onward, was commemorated in literary works from John Milton to Jonathan Edwards and William Blake. Bruegel approached the subject with his usual good humor, creating as a result a new version of Pandemonium. And moving away from biblical judgments, he fashioned for the good angels to remedy not so much a theological crisis as a "fine kettle of fish."

While Bosch may be credited for inspiring this venture into the fantastic, Bruegel moved away from exotic ethereal creatures in favor of more earthy beasts with facial expressions, peering eyes, human limbs. These are no phantoms glowing in the painter's imagination. They are pests—annoying, grotesque and interfering, prickly, buzzing, mocking, biting, threatening—even as they fall in total disarray into the depths of darkness. To make the scene more intriguing, Bruegel throws in realistically drawn detail: butterfly wings, an embellished robe, a grinning possum, a musical instrument or two, giving the scene more credibility and the fallen a certain wicked charm.

Fierce creatures as a metaphor for evil abound in literature. "Tyger! Tyger! Burning bright / in the forests of the night," wrote William Blake (1757–1827) in Songs of Experience, "What immortal hand or eye / could frame thy fearful symmetry?" These famous lines generated countless discussions and interpretations, many about the possible link between Blake's legendary carnivore and the struggle between good and evil in the world, between the tiger and "man in his fallen state." "When the stars threw down their spears, / And watered heaven with their tears, / Did he smile his work to see? / Did he who made the lamb make thee?"

Lit by heaven, angels in flowing robes with the archangel in the lead, his princely gown billowing, are swatting dozens of monsters, while still more are hatching. Though outnumbered, the angels seem unfazed. The proceedings provide a tantalizing spectacle, not just of failed angels but of all monsters, among them nature's ferocious bloodsucking nation of fleas, ticks, mosquitoes, and other horrors, pestering without provocation and spreading pain and disease, from epidemic typhus to bartonellosis, from rickettsioses to relapsing fever.

"I dried my tears and armed my fears / with ten thousand shields and spears," wrote Blake in Songs of Experience, lamenting the loss of a guardian angel and building up personal defenses and strength. When it comes to swatting monsters in today's infectious Bedlam, we can do no less. Like Bruegel's angels we are outnumbered. Either we remain vigilant and keep the pests at bay, or we join them and fall.

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Increase in Pneumococcus Macrolide Resistance, United States

Dengue Hemorrhagic Fever in French Polynesia, 2001

Serologic Evidence of Frequent Human Infection with WU and KI Polyomaviruses

Increased Toxin Production in *Bordetella pertussis* Strains and Pertussis Resurgence

Molecular Epidemiology of Rabies in Southern China

Porcine Prion Protein and Susceptibility to BSE and Atypical Scrapie

Strategy to Enhance Influenza Surveillance Worldwide

Invasive Group B Streptococcal Disease in the Elderly, Minnesota, 2003–2007

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

August 3–4, 2009
UC Berkeley CIDER SUMMIT 2009: "Advances in the Control and Epidemiology of Emerging Infectious Diseases"
Sheraton Gateway Hotel
Burlingame, CA, USA
http://www.idready.org/CIDERSummit2009.html

August 10–21, 2009
11th International Dengue Course
http://www.ipk.sld.cu/cursos/dengue2009/indexen.htm

August 29–September 2, 2009
Infectious Disease 2009 Board Review Course
14th Annual Comprehensive Review for Board Preparation
McLean, VA, USA
http://www.cbcbiomed.com

October 29–November 1, 2009
47th Annual Meeting of IDSA and HIVMA
Philadelphia, PA, USA
http://www.idsociety.org/Content.aspx?id=12006

November 7–11, 2009
American Public Health Association's 137th Annual Meeting and Exposition
Philadelphia, PA, USA
http://www.apha.org/meetings

November 18–22, 2009
American Society of Tropical Medicine and Hygiene 58th Annual Meeting
Marriott Wardman Park
Washington, DC, USA
http://www.astmh.org/meetings/index.cfm

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). In 50–150 words, describe timely events of interest to our readers. Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions and earn continuing medical education (CME) credit, please go to **http://www.medscape.com/cme/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.com. If you are not registered on Medscape.com, 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 is acceptable as evidence of participation in CME activities. If you are not licensed in the US and want to obtain an AMA PRA CME credit, please complete the questions online, print the certificate and present it to your national medical association.

Article Title

Cluster of Sylvatic Epidemic Typhus Cases Associated with Flying Squirrels, 2004–2006

CME Questions

1. Which of the following statements about the transmission of sylvatic epidemic typhus (ST) is most accurate?

A. It is most common in the southern United States
B. It has been established that transmission to humans occurs after arthropod bites
C. It is transmitted only by feces from flying squirrels
D. Contact with flying squirrels or their nests has been confirmed in only a minority of cases

2. What was the most likely common exposure to *Rickettsia prowazekii* in the current case series?

A. All case-patients had done extensive cave explorations
B. All case-patients lived in a cabin with flying squirrel nests in the walls
C. All case-patients had led daily nature walks through the surrounding woods
D. All case-patients had performed nature shows involving live animals

3. Which of the following symptoms and signs of ST was least evident in the current case series?

A. Rash
B. Fever
C. Headache
D. Malaise

4. Which of the following statements about the management of suspected ST is most accurate?

A. Treatment may begin without laboratory confirmation of infection
B. Serologic confirmation of infection is based on a 10-fold increase in immunoglobulin (Ig) G titers or more
C. IgM antibodies to *R. prowazekii* always decrease rapidly following acute infection
D. PCR analysis for *R. prowazekii* is now widely available

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 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 www.cdc.gov/eid/ncidod/ EID/instruct.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (www.cdc. gov/ncidod/EID/trans.htm).

Instructions to Authors

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, figure legends, appendixes, and figures. Each figure should be in a separate file.

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. Include up to 10 keywords; use terms listed in Medical Subject Headings Index Medicus.

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. Provide figures as separate files, not embedded in MS Word. Use Arial font for text content. Place keys within figure area. Provide footnotes and other information (e.g., source/copyright data, explanation of boldface) in figure legend. Submit figures with text content in native, editable, PC file formats (e.g., MS Excel/PowerPoint). Submit image files (e.g., electromicrographs) without text content as high-resolution (300 dpi/ppi minimum) TIFF or JPG files. Submit separate files for multiple figure panels (e.g., A, B, C). EPS files are admissible but should be saved with fonts embedded (not converted to lines). No PNG or BMP files are admissible. For additional guidance, contact fue7@cdc.gov or 404-639-1250.

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Types of Articles

Perspectives. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief biographical sketch. Articles in this section should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may also 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Synopses. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief 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. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research Studies. Articles should be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary, and a brief 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 be under 3,500 words and should include references, not to exceed 40. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), a one-sentence summary of the conclusions, and a brief 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 a brief biographical sketch. Dispatches are updates on infectious disease trends and research. The articles 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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no figures or tables.

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.

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.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Name, publisher, number of pages, other pertinent details should be included.

Announcements. We welcome brief announcements (50–150 words) of timely events of interest to our readers. (Announcements may be posted online only, depending on the event date.)

Conference Summaries. Summaries of emerging infectious disease conference activities are published online only. Summaries, which should contain 500–1,000 words, should focus on content rather than process and may provide illustrations, references, and links to full reports of conference activities.