EMERGING INFECTIOUS DISEASES®

July 2013

Vector-Borne Infections

Charles E. Burchfield (1893–1967) The Insect Chorus (1917)
Opaque and transparent watercolor with ink, graphite, and crayon on off-white paper (50.8 cm x 38.1 cm [40 x 30]) Munson-Williams-Proctor Arts Institute, Museum of Art, Utica, New York, Edward W. Root Bequest, 1957

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INFECTIOUS DISEASES®

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On the Cover

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About the Cover p. 1184

Synopsis

Medscape EDUCATION ACTIVITY

Transmission of *Streptococcus equi* Subspecies *zooepidemicus* Infection from Horses to Humans 1041
S. Pelkonen et al.
This agent may cause severe illness in humans and should be considered an emerging zoonosis.

p. 1050

Research

Travel-associated Illness Trends and Clusters, 2000–2010..... 1049
K. Leder et al.
Enteric fever, dengue, and rabies are increasing; malaria is decreasing; and malaria, dengue, and enteric fever were detected in clusters.

Quantifying Effect of Geographic Location on Epidemiology of *Plasmodium vivax* Malaria..... 1058
A.A. Lover and R.J. Coker
Regional variations should be considered in patient care, surveillance, and control programs.

p. 1100

Mutation in Spike Protein Cleavage Site and Pathogenesis of Feline Coronavirus..... 1066
B.N. Licitra et al.
Knowledge of proteolytic requirement modifications may facilitate development of novel strategies against human strains.

Pneumococcal Serotypes before and after Introduction of Conjugate Vaccines, United States, 1999–2011..... 1074
S.S. Richter et al.
Use of the 7-valent vaccine dramatically reduced prevalence of its serotypes.

Influence of Pneumococcal Vaccines and Respiratory Syncytial Virus on Alveolar Pneumonia, Israel 1084
D.M. Weinberger et al.
Seasonal variations in RSV activity masked the benefit of 7-valent vaccines.

Dispatches

1092 Avian Metapneumovirus Subgroup C Infection in Chickens, China
L. Wei et al.

1095 Human Alveolar Echinococcosis in Kyrgyzstan
J. Usubalieva et al.

1098 Molecular Epidemiologic Source Tracking of Orally Transmitted Chagas Disease, Venezuela
M. Segovia et al.

EMERGING INFECTIOUS DISEASES
July 2013

1102 **Unique Clone of *Coxiella burnetii* Causing Severe Q Fever, French Guiana**
A. Mahamat et al.

1105 ***Babesia microti* Infection, Eastern Pennsylvania, USA**
M.E. Perez Acosta et al.

1108 **Reemergence of Chikungunya Virus in Bo, Sierra Leone**
R. Ansumana et al.

1111 **Novel *Bartonella* Agent as Cause of Verruga Peruana**
D.L. Blazes et al.

1115 **Schmallenberg Virus among Female Lambs, Belgium, 2012**
F. Claine et al.

1118 ***Psychrobacter arenosus* Bacteremia after Blood Transfusion, France**
Y. Caspar et al.

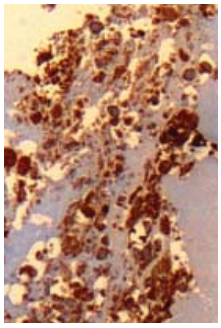
1121 **Ciprofloxacin-Resistant *Campylobacter* spp. in Retail Chicken, Western Canada**
A. Agunos et al.

1125 **Asynchronous Onset of Clinical Disease in BSE-Infected Macaques**
J. Montag et al.

1128 **Prevalence of Nontuberculous Mycobacteria in Cystic Fibrosis Clinics, United Kingdom, 2009**
P. Seddon et al.

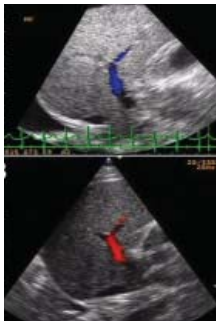
1131 **Reducing Visceral Leishmaniasis by Insecticide Impregnation of Bed-Nets, Bangladesh**
D. Mondal et al.

1135 **Genetic Variants of *Orientia tsutsugamushi* in Domestic Rodents, Northern China**
M. Zhang et al.



p. 1103

p. 1162



1138 **Undetected Multidrug-Resistant Tuberculosis Amplified by First-line Therapy in Mixed Infection**
S.M. Hingley-Wilson et al.

1142 **Clinical Findings for Early Human Cases of Influenza A(H7N9) Virus Infection, Shanghai, China**
S. Lu et al.

1147 **Multidrug-Resistant Atypical Variants of *Shigella flexneri* in China**
S. Qiu et al.

Letters

1151 **MDR TB Transmission, Singapore**

1152 **Human Infection with Marten Tapeworm**

1155 ***Campylobacter jejuni* in Hospitalized Patients with Diarrhea, Kolkata, India**

1156 **Verona Integron–encoded Metallo-β-Lactamase 1 in Enterobacteria, Ontario, Canada**

1159 **Novel Bat-borne Hantavirus, Vietnam**

1161 **Possible Cause of Liver Failure in Patient with Dengue Shock Syndrome**

1163 **Spotted Fever Group Rickettsiae in Questing Ticks, Central Spain**

1165 **Neonatal *Granulicatella elegans* Bacteremia, London, UK**

EMERGING INFECTIOUS DISEASES
July 2013

1167 *Bartonella* Species in Raccoons and Feral Cats, Georgia, USA

1168 Rifampin-Resistant *Mycobacterium bovis* BCG–Induced Disease in HIV-Infected Infant, Vietnam

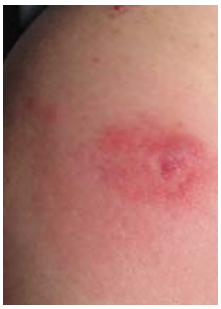
1170 Novel *Bulleidia extructa* Periprosthetic Hip Joint Infection, United States

1171 Tick-borne Encephalitis Virus, Zealand, Denmark, 2011

1173 Usutu Virus in Migratory Song Thrushes, Spain

1176 *Rickettsia aeschlimannii* Infection, Greece

1177 Antibodies against Rift Valley Fever Virus in Cattle, Mozambique



p. 1176

1179 Avian Influenza A(H7N9) Virus Infections, Shanghai, China

Books and Media

1182 The Stealth Virus
J.W. Tang

About the Cover

1184 Summer Buzz

Etymologia

1181 Verona Integron

Online Report

Influence of Humans on Evolution and Mobilization of Environmental Antibiotic Resistome

http:/dx.doi.org/10.3201/eid1907.120871

Transmission of *Streptococcus equi* Subspecies *zooepidemicus* Infection from Horses to Humans

Sinikka Pelkonen,[1] Susanne B. Lindahl,[1] Päivi Suomala, Jari Karhukorpi, Sakari Vuorinen, Irma Koivula, Tia Väisänen, Jaana Pentikäinen, Tiina Autio, and Tamara Tuuminen

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Release date: June 28, 2013; Expiration date: June 28, 2014

Learning Objectives

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

- Evaluate the clinical presentation and outcomes of patients with *Streptococcus equi zooepidemicus* infection
- Analyze the transmission of *S. zooepidemicus*
- Distinguish molecular characteristics of *S. zooepidemicus*

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[1]These authors contributed equally to this article.

Streptococcus equi subspecies *zooepidemicus* (*S. zooepidemicus*) is a zoonotic pathogen for persons in contact with horses. In horses, *S. zooepidemicus* is an opportunistic pathogen, but human infections associated with *S. zooepidemicus* are often severe. Within 6 months in 2011, 3 unrelated cases of severe, disseminated *S. zooepidemicus* infection occurred in men working with horses in eastern Finland. To clarify the pathogen's epidemiology, we describe the clinical features of the infection in 3 patients and compare the *S. zooepidemicus* isolates from the human cases with *S. zooepidemicus* isolates from horses. The isolates were analyzed by using pulsed-field gel electrophoresis, multilocus sequence typing, and sequencing of the *szP* gene. Molecular typing methods showed that human and equine isolates were identical or closely related. These results emphasize that *S. zooepidemicus* transmitted from horses can lead to severe infections in humans. As leisure and professional equine sports continue to grow, this infection should be recognized as an emerging zoonosis.

Streptococcus equi subspecies *zooepidemicus* (*S. zooepidemicus*) is a β-hemolytic, Lancefield group C streptococcal bacterium. *S. zooepidemicus* is considered an opportunistic commensal in horses (*1*–*3*), but it may also cause infections in other domestic animals such as cattle, sheep, goats, pigs, dogs, and cats (*4*–*10*). Another subspecies of the same genus, *Streptococcus equi* subsp. *equi* (*S. equi*), causes strangles, the highly contagious and serious disease in horses (*1*,*11*,*12*).

S. zooepidemicus shares >98% DNA sequence homology with *S. equi* (*1*) and >80% DNA sequence homology with *Streptococcus pyogenes* (*13*), a Lancefield group A streptococcus and major human pathogen. Although considered an opportunistic pathogen, *S. zooepidemicus* shares important virulence factors with both *S. equi* and *S. pyogenes* such as the M-like proteins, superantigens (sAgs), and the presence of a hyaluronic acid capsule in certain strains. The variable M-protein, located on the surface of *S. pyogenes*, can be used to differentiate *S. pyogenes* strains serologically where the M1 serotype is associated with invasive disease in humans (*14*). The M-like, cell-wall–anchored surface protein SzP, found in all strains of *S. zooepidemicus*, is essential for the pathogenesis of the disease, at least in horses, where it binds fibrinogen and exhibits antiphagocytic activity that impairs with host protection. The sAgs SeeH, SeeI, SeeL, and SeeM found in *S. equi* share 96%–99% amino acid sequence homology with *S. pyogenes* sAgs SpeH, SpeI, SpeL, and SpeM (*15*). However, few investigated strains of *S. zooepidemicus* contain homologs to these sAgs. Instead, novel sAgs (SzeF, SzeN, and SzeP) have been identified in certain strains, sharing 34%–59% homology with SpeH, SpeM, and SpeL of *S. pyogenes* (*16*).

S. zooepidemicus has seldom been isolated from humans. Surprisingly, most published data on humans go back to the latter part of the 1980s (*17*). Occasional human infection was reported as a result of the consumption of homemade cheese or unpasteurized milk from cows with mastitis (*17*). In humans, *S. zooepidemicus* may cause glomerulonephritis and rheumatic fever, which are known sequelae of *S. pyogenes* (group A) infections (*18*). Meningitis and purulent arthritis have also been reported (*19*,*20*).

S. zooepidemicus displays a wide genetic variation between different isolates (*13*,*21*–*23*). The sequence of the SzP protein gene (*szP*) has been shown to vary greatly between different strains of *S. zooepidemicus* (*24*–*26*), and the variable regions of *szP* can be used to genetically differentiate strains within the subspecies (*27*–*29*). Pulsed-field gel electrophoresis (PFGE) is a DNA-based typing technique that is highly discriminatory and has been used in epidemiologic investigations of *S. zooepidemicus* outbreaks (*30*,*31*).

Multilocus sequence typing (MLST) is a method for characterization of bacterial isolates by comparing sequences of several gene fragments. Webb et al. (*22*) developed a MLST protocol for *S. zooepidemicus* consisting of 7 housekeeping genes. Obtained sequences are compared to previously deposited sequences, and a sequence type (ST) is assigned from the online PubMLST *S. zooepidemicus* database (http://pubmlst.org/szooepidemicus) developed by Jolley et al. (*32*).

Within a 6-month period, through our routine practice, we found 3 cases of severe disseminated disease in humans caused by *S. zooepidemicus*. The purpose of this study was to 1) characterize the clinical presentation of the disease caused by *S. zooepidemicus*, 2) microbiologically characterize the isolated strains, and 3) identify clonality of human isolates for comparison to equine isolates from contact horse stables and other horse farms of the surrounding area.

Patient 1

A 57-year-old man, a farmer and horse breeder from central Finland, was admitted unconscious and febrile to the emergency room of a principal hospital in February of 2011. Cerebrospinal fluid (CSF) was collected, and he was referred to the intensive care unit. He had aortic valve insufficiency and had been catheterized 3 months earlier. His condition was septic, with clinical symptoms of meningismus and pulmonary congestion. The C-reactive protein (CRP) level was 564 mg/L (reference <3 mg/L) and the leukocyte count 15.9×10^9 cells/L (reference 3.4–8.2 $\times 10^9$ cells/L). Microscopy staining of the CSF revealed gram-positive cocci in chains with a considerable number of polymorphonuclear cells. The next day, bacteria subsequently identified as *S. zooepidemicus* (Table 1) grew from the CSF and 4 of the 4 blood culture bottles, leading to a primary diagnosis of meningitis and sepsis. Intravenous high-dose penicillin treatment (5 weeks) was started

Table 1. Bacteriologic identification of *Streptococcus equi* subspecies *zooepidemicus* isolates from patient samples in different ISLAB laboratories*

Isolate (patient, source)	APIStrep†	STR Rapid ID32†	Agglutination with group serum specimens	VITEK2 GP-ID†	AccuProbe
Hum1 (patient 1, CSF)	0063607 (99.9% *S. zooepidemicus)*	15412061151 (99.9% *S. zooepidemicus)*	Group C	Not done	Not done
Hum2 (patient 2, blood)	Not done	15412061151 (99.9% *S. zooepidemicus)*	Group C	053450364317451 (99% *S. zooepidemicus)*	*Streptococcus agalactiae*‡
Hum3 (patient 3, abdominal aortic wall)	Not done	15512061111 (99.9% *S. zooepidemicus)*	Group C	Not done	Not done

*ISLAB, Eastern Finland Laboratory Centre Joint Authority Enterprise; CSF, cerebrospinal fluid.
†bioMérieux, Marcy l'Etoile, France.
‡*S. agalactiae* (315,588 reflective light units, the reference range below 50,000 reflective light units*). S. agalactiae* and *S. zooepidemicus* are known to cross-react in the AccuProbe Group B Streptococcus Culture ID Test (Gen-Probe, San Diego, CA, USA).

in combination with gentamicin (first 10 days). Two and a half days after admission, the patient regained consciousness. Intravascular coagulopathy developed, and 20 days later, progressive endocarditis. The bicuspid native aortic valve was resected the same day, and several bacterial patches were observed. His perioperative blood cultures remained negative. Neurologic sequelae did not develop, but his recovery and rehabilitation required several weeks.

Patient 2

A 62-year-old-man, a truck driver and horse trainer from eastern Finland, returned home from work in a febrile and confused state in May 2011. The next day, on hospital admission, he had pain and swelling of the right knee and right shoulder. He was hyperglycemic and had untreated non–insulin-dependent diabetes mellitus. The synovial fluid aspirated from his knee was turbid, with a leukocyte count of 86.0×10^9 cells/L and a high percentage of polymorphonuclear cells (87%). The CRP level was 329 mg/L and the blood leukocyte count was 19.3×10^9 cells/L. Antimicrobial drug therapy with intravenous cefuroxime was started. The next day, bacteria subsequently identified as *S. zooepidemicus* were cultured from his right knee and 4 of 4 blood culture bottles (Table 1). On the third day, arthroscopic synovectomy and irrigation of the right knee was performed, and the procedure was repeated. Cefuroxime was changed to intravenous vancomycin without therapeutic response. The CRP level remained high (229 mg/L) and the leukocyte count was 15.3×10^9 cells/L. Next, a combination of penicillin G with clindamycin was administered. He had no evidence of endocarditis, but Tc99m scintigraphy revealed an uptake in the patient's right shoulder and lower jaw region. Arthroscopic debridement and irrigation of the right shoulder were performed, and purulent synovial fluid was collected for culture. Antimicrobial drug therapy continued with intravenous cefuroxime and clindamycin for 2 weeks; thereafter, with oral cephalexin and clindamycin for 1 week. The patient's clinical condition gradually improved, and finally, he was able to walk with crutches. He was discharged from the hospital 6 weeks after the onset of illness.

Patient 3

A 49-year-old man, a horse trainer from eastern Finland, was admitted to the hospital in August 2011 because of severe, prolonged low-back pain. A horse had kicked his forehead 2–3 weeks earlier. The accident did not require medical attention at that time; however, the low-back pain had increased gradually. He had medicated himself with ibuprofen, 400 mg up to 20 tablets per day, without relief, except when lying supine. He did not record his temperature but was sweating after taking ibuprofen and sought medical attention when walking became difficult. His condition was treated as muscle pain. After a week he returned to the medical center because of excruciating pain in his back. There were no abnormal radiologic findings. On clinical examination, he was nonfebrile and had no clinical symptoms or hemodynamic abnormalities. The clinical findings were unremarkable, except for the pain in his lower back on percussion and a pulsating abdominal mass. Laboratory tests showed leukocytosis (16.2×10^9 cells/L), an elevated erythrocyte sedimentation rate of 73 mm/h (reference 1–15 mm/h), and an elevated CRP level of 217 mg/L. Computed tomography revealed a psoas abscess (65 × 35 × 30 mm) linked to an infected aortic aneurysm (diameter 40 mm). The aneurysm was resected and replaced by a Y-prosthesis, and the psoas abscess was drained. Gram stain of tissue obtained through operation on the abdominal aorta and debridement of the psoas abscess revealed gram-positive cocci in 3 (2 from the aortic wall and 1 from the psoas abscess) of the 4 samples. The patient's condition was treated with piperacillin-tazobactam, later replaced with intravenous penicillin. Transesophageal echocardiography showed no signs of endocarditis. The patient recovered without sequelae.

Materials and Methods

Microbiological Diagnostics of *S. zooepidemicus* Strains in Clinical Laboratories

Each clinical laboratory used the standard operating procedures and standard culture media of their own. CSF and synovial fluid samples were cultured on blood and/or

chocolate agar and blood samples in blood culture bottles and incubated aerobically and anaerobically. For identification, Gram stain and agglutination with streptococcal group sera (Streptococcal Grouping Kit; Oxoid Ltd., Basingstoke, UK) were carried out in all laboratories. The identification of *S. zooepidemicus* to the species level varied between the laboratories, and was performed using at least one of the following tests as shown in Table 1: APIStrep, STR Rapid ID32, or VITEK2 GP-ID (all from bioMérieux Marcy l'Etoile, France), combined with AccuProbe Group B Streptococcus Culture ID Test (Gen-Probe, San Diego, CA, USA).

Antibiotic Susceptibility of Human Isolates

The antibiotic susceptibility profiles were studied with the disk diffusion method (patients 1 and 3) or Etest (patient 2). Results were interpreted according to the EUCAST rules (www.eucast.org/eucast_disk_diffusion_test/breakpoints/).

Collection and Microbiological Characterization of Equine Isolates

None of the horses from the stables associated with the first 2 human cases (patients 1 and 2) showed any signs of respiratory illness. The horses from the third stable (owned by patient 3) were not examined; however, the owner did not recall any clinical signs of respiratory or other disease in his horses. Nasal swab specimens were collected from 7 horses owned by patient 1 (stable A) and 4 horses owned by patient 2 (stable H). The swabs were streaked onto bovine blood agar plates and incubated in a 5% CO_2 atmosphere at 37°C (according to the standard operating procedures of the Finnish Food Safety Authority Evira, Kuopio, Finland) for 24 hours. β-hemolytic colonies were studied with conventional methods, and biochemical characterization was performed by using Rapid ID32 Strep (bioMérieux). *S. zooepidemicus* was isolated from 5 horses in stable A, but not from any horse in stable H. Six other *S. zooepidemicus* isolates from horses unrelated to the described human cases (stables B to F) (Table 2) were included in the genetic comparison.

Pulsed-field Gel Electrophoresis

Three human isolates (1 from each patient) and 11 equine isolates of *S. zooepidemicus* were investigated by PFGE, sequencing of the *szP* gene, and MLST. DNA isolation was performed as described by Elliot et al. (*33*), and 40 U of *Sma*I was used for digestion. The chromosomal digests were separated by PFGE, with a switch time of 5 to 40 s for 20 h at a 120° angle and a voltage gradient of 6 V/cm at 12°C. Chromosomal DNA of *Salmonella enterica* serovar Braenderup H9182 was used as a marker.

Sequencing of the *szP* gene and MLST

Isolates of *S. zooepidemicus* were cultured on 5% horse blood agar (National Veterinary Institute, Uppsala, Sweden) in a 5% CO_2 atmosphere at 37°C for 24 h. Preparation of DNA from bacterial culture was performed by a boiling procedure; a 1-μL loop of bacteria was suspended in 100 μL of sterile H_2O and incubated at 98°C for 15 min. The samples were centrifuged and the supernatants were collected and used as templates in the sequencing analyses.

The isolates of *S. zooepidemicus* (n = 14) were investigated by sequencing a 373-bp fragment of the SzP protein gene (*25*). Sequencing was performed according to Båverud et al. (*34*). Sequences were edited, assembled, and analyzed by using BioNumerics 6.5 (Applied Maths, Saint-Martens-Latem, Belgium).

MLST was performed according to Webb et al. (*22*). Sequences were edited, assembled, and analyzed by using BioNumerics 6.5. Sequence types (STs) were determined using the PubMLST *S. zooepidemicus* database.

Results

Microbiological Identification and Antibiotic Susceptibility of Human Isolates

The colonies of *S. zooepidemicus* on blood agar were large and mucoid and had a wide zone of β-hemolysis. All isolates were sensitive to erythromycin, clindamycin, penicillin, vancomycin, and cephalexin (data not shown). Microbiological identification data for the *S. zooepidemicus* isolates from human cases are shown in Table 1.

Molecular Characterization of Isolates

The *S. zooepidemicus* isolates displayed 10 ST types by MLST. Their relatedness was compared by using eBurst (http://eburst.mlst.net) of all MLST STs for *S. equi* subsp. *zooepidemicus* and *S. equi* subsp. *equi* recorded in the PubMLST *S. zooepidemicus* database (February 7, 2013) (Figure 1, Appendix, wwwnc.cdc.gov/EID/article/19/7/12-1365-F1.htm). eBurst analysis indicated that ST-10, displayed by 3 isolates, Hum1, Hum2, and equine 648/11, was not related to any other STs of the *S. zooepidemicus* isolates examined in this study (Figure 1; Table 2). ST-209 and ST-201 are double-locus variants (DLVs) and were displayed by Hum3 isolate and horse isolate 6939/10, respectively. All other detected STs were unrelated to each other. Isolates from stables E (8110/09) and F (7723/09) displayed STs not previously described in the PubMLST *S. zooepidemicus* database. In addition, no product was obtained from forward and reverse primers for the *yqiL* gene from the isolate from stable F (7723/09).

The human isolates Hum1 (patient 1) and Hum2 (patient 2) displayed an *szP* sequence (GenBank accession no. AF519489) and MLST sequence type (ST-10) identical to the equine isolate 648/11 (stable A) (Table 2). Hum1 was also identical to equine isolate 648/11 on PFGE (Figure 2). Hum2, however, differed from Hum1 and 648/11 by 6

bands on the PFGE profile. The third human isolate, Hum3 (patient 3), was closely related to 1 equine isolate (6939/10) from an unrelated stable (stable D). These isolates displayed an identical *szP* sequence (accession no. AF519488). Their PFGE profiles were almost identical, and the MLST types ST-209 (Hum3) and ST-201 (6939/10) were DLVs. None of the other equine isolates displayed the same *szP* sequence type or MLST STs as the human isolates. Among the 5 *S. zooepidemicus* isolates from stable A, 645/11 was identical to 647/11 on the basis of the MLST ST (ST-175), *szP* type (II), and PFGE profile. All other isolates differed from each other. Several equine isolates displayed *szP* sequences not previously described in GenBank (645/11, 646/11, 647/11, and 1128). All *szP* sequencing results and corresponding GenBank accession numbers are listed in Table 2.

Discussion

We report 3 unrelated cases of *S. zooepidemicus* infection in patients from eastern Finland who had close and continuous contact with horses. It is noteworthy that the disease in all 3 patients was invasive and severe, requiring prolonged treatment and rehabilitation. Sepsis occurred in 2 cases (patients 1 and 2), meningitis and endocarditis in 1 (patient 1), purulent arthritis in 1 (patient 2), and a psoas abscess in connection with an aortic wall infection in 1 (patient 3). In patient 3, transient bacteremia might have occurred earlier.

MLST, PFGE, and sequencing of the SzP protein gene demonstrated identical profiles in a human isolate (Hum1) with an equine isolate (648/11), which strongly supports the zoonotic nature of this disease. Notably, the strain (ST-10) colonized the horse's nostrils and acted as an innocent commensal, whereas in humans this strain appeared highly virulent and caused severe illness. In the second case (patient 2), we were unable to isolate the same strain from his horses. This failure may have been due to a transient *S. zooepidemicus* carriage in the nasopharynx, lymphoid tissues, or respiratory tract of the horse. Patient 2 might have been in contact with other horses as well. The strains from patient 1 and patient 2 were identical according to both *szP* sequencing and MLST, which supports the close relationship between the Hum1 and Hum2 isolates, and although the 2 isolates differed on PFGE analysis (Figure 1), the data strongly suggest that the infection of patient 2 was also transmitted zoonotically. ST-10 is a single-locus variant (SLV) of ST-72, which previously has been isolated from a case of human nephritis in the UK in 1983 (http://pubmlst.org/szooepidemicus/), and from a large outbreak of severe human nephritis in Brazil during 1997 and 1998 associated with consumption of unpasteurized cheese (*13,35,36*). The isolated strain in the Brazil outbreak was shown to have several genetic similarities to group A streptococci (*35*).

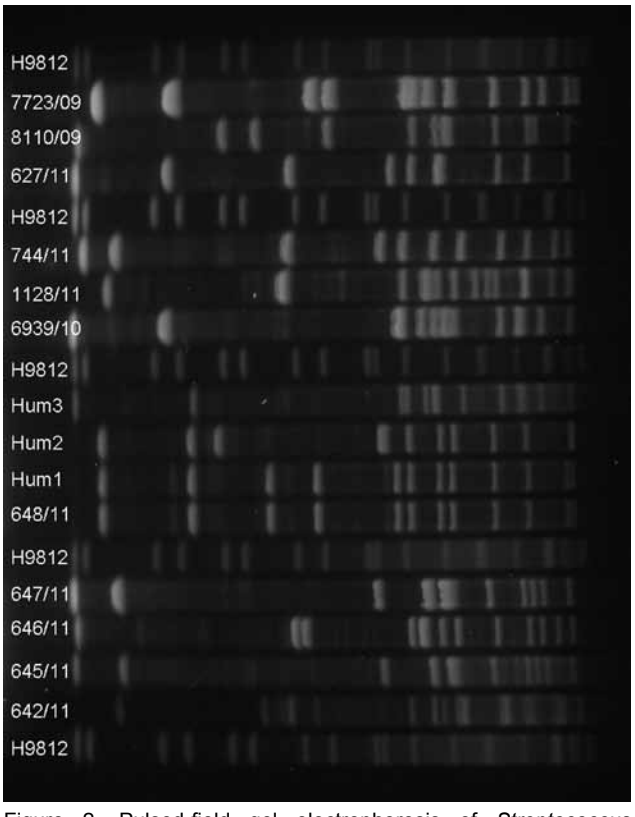

Figure 2. Pulsed-field gel electrophoresis of *Streptococcus equi* subspecies *zooepidemicus* isolates using *Sma*I. The lanes are marked with the number of the respective isolate. DNA of *Salmonella enterica* serovar Braenderup H9182 was used as a molecular marker.

PFGE reveals random genetic events, such as point mutations or insertions or deletions of genetic material (*37*), thereby being often a more sensitive method than MLST to identify recent epidemic strains. However, it is not possible to estimate whether *S. zooepidemicus* isolates from patients 1 and 2, with altered PFGE profiles and approximately a 3-months' gap between the diagnoses of disease, could be of the same origin. Notably, the strain isolated from patient 3 (Hum3), which differed completely from Hum1 and Hum2, was identical by MLST (ST-209) to a strain isolated from horses in an outbreak of respiratory disease in Iceland in 2010 (Bjornsdottir et al., unpub data). The Hum3 isolate also shared a *szP* sequence type (accession no. AF519488) previously found in horses with respiratory disease (S.B. Lindahl, unpub. data) as well as in an asymptomatic horse (6939/10) in this study. The ST-209 strain has further been isolated from a person with septicemia (that was associated with abortion) in Iceland in 2010, and has a SLV (ST-200) and a DLV (ST-201) that have been reported from cases of abortion/uterine infections in horses (http://pubmlst.org/szooepidemicus/). The ST-201 was also found in one of the healthy horses in this study (Table 2).

Table 2. Molecular characterization of *Streptococcus equi* subspecies *zooepidemicus* isolates by sequencing of the SzP protein gene and by multilocus sequence typing*

Isolate ID no.	Origin	Year	Stable	MLST sequence type	SzP type	SzP GenBank accession no.
Hum1	Patient 1, blood	2011	A	ST-10	I	AF519489
Hum2	Patient 2, blood	2011	H	ST-10	I	AF519489
Hum3	Patient 3, aortic wall	2011	Not done	ST-209	VII	AF519488
642/11	Horse, nasal swab, nonclinical	2011	A	ST-147	IV	AF519482
645/11	Horse, nasal swab, nonclinical	2011	A	ST-175	II	KC287220†
646/11	Horse, nasal swab, nonclinical	2011	A	ST-66	V	KC287221†
647/11	Horse, nasal swab, nonclinical	2011	A	ST-175	II	KC287220†
648/11	Horse, nasal swab, nonclinical	2011	A	ST-10	I	AF519489
744/11	Horse, nasal swab, nonclinical‡	2011	C	ST-80	VIII	U04620
1128/11	Horse, foal, sepsis	2011	B	ST-5	VI	KC287222†
627/11	Horse, nasal swab, nonclinical‡	2011	C	ST-115	III	AF519478
6939/10	Horse, nasal swab, nonclinical‡	2010	D	ST-201	VII	AF519488
8110/09	Horse, synovial fluid (arthritis)	2009	E	ST-299†	III	AF519478
7723/09	Horse, foal, tracheal fluid, respiratory infection§	2009	F	ST-XX†¶	III	AF519478

*ID, identification; MLST, multilocus sequence typing.
†Not previously described.
‡The samples were collected for screening of *S. equi* subsp. *equi,* but *S. equi* subsp. *zooepidemicus* was identified.
§Co-infection with *Pasteurella* sp. and *S. suis.*
¶Recorded in the PubMLST database: 8 (arcC)–52 (nrdE)–2 (proS)–14 (spi)–1 (tdk)–22 (tpi)–n/a (*yqiL*).

All strains of *S. zooepidemicus* displayed mucoid colonies on the agar plates, indicating expression of a hyaluronic acid capsule, a well-known virulence factor in other pathogenic streptococci, such as *S. equi* in horses and *S. pyogenes* in humans. However, the expression of the mucoid capsule was variable: Hum1 strain produced large and highly mucoid colonies, whereas those from Hum3 were heterogeneous in colony size and less mucoid. Whether there is a correlation between the production of mucinous substance and severity of the disease remains to be determined. Additional virulence factors, such as the presence of sAgs (*16*), would be intriguing to investigate. In *S. pyogenes*, variation in the M-protein is attributed to variable virulence. For example, the M1 strains are the most pathogenic (*14*). The sequence variants of the SzP protein gene in *S. zooepidemicus* were investigated but could not be correlated with clinical features in horses in a study by Walker and Runyan (*26*). However, determining such a correlation might be possible for the human isolates.

Recently, an outbreak of invasive *S. zooepidemicus* infection has been reported from Finland by Kuusi et al. (*30*). Altogether, 7 patients were identified: 6 had septicemia and 1 had purulent arthritis. All had consumed goat cheese produced from unpasteurized milk in a small-scale dairy. In Finland (population 5.2 million), all invasive streptococcal infections must be reported to the National Infectious Disease Register. As reviewed by Kuusi et al., only 3 cases of invasive *S. zooepidemicus* infections were reported to the register from 1992 through 2002, and ≈10 cases of invasive group C streptococcal infections occurred annually. In other words, even invasive isolates were often typed only to the Lancefield group level.

The novelty of our investigation is that an identical *S. zooepidemicus* strain was isolated from patient 1 and from a healthy horse in his stable, suggesting zoonotic transmission. Furthermore, patient 2 was infected with a *S. zooepidemicus* strain clonally related to that of patient 1, as judged by 2 independent typing methods, although patients 1 and 2 lived 140 km apart without a verified contact with each other. Notably, the isolate from patient 1 was highly virulent in humans but did not cause any clinical infection in the horse. In contrast, the isolate from patient 3 had the same MLST type as the strain previously isolated from several horses in an outbreak of respiratory disease. Our work yielded 3 new sequences of the *szP* gene, deposited under GenBank accession nos. KC287220 (isolate 645/11), KC287221 (isolate 646/11), and KC287222 (isolate 1128/11). Further, isolate 8110/09 was added to the PubMLST *S. zooepidemicus* database with ST-299. The isolate 7723/09 could not be assigned a ST because there was no product for the *yqiL* gene; however, the isolate is recorded in the PubMLST *S. zooepidemicus* database with the following allele sequence: 8 (arcC)–52 (nrdE)–2 (proS)–14 (spi)–1 (tdk)–22 (tpi)–n/a (*yqiL*).

Conclusions

Leisure and professional equine sports activities are growing in many countries. *S. zooepidemicus* infection transmitted from horses may cause severe illness in humans and should be considered an emerging zoonosis. Bacteriological identification of *S. zooepidemicus* is cheap and feasible with simple fermentation methods. Therefore, typing to the species level is strongly recommended for all clinical laboratories whenever group C streptococci are recovered from severely infected persons. Early identification of *S. zooepidemicus* will facilitate appropriate medical intervention and timely epidemiologic surveillance and finally, prevent the spread of a potentially life-threatening pathogen.

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T.A., S.B.L., and T.T. conceived and wrote the first draft; P.S., T.T., J.K. isolated the human strains; T.A. and S.P. isolated the equine strains; S.V., I.K., T.V., and J.K. processed patient records; S.B.L. performed the SzP sequencing and MLST analyses; S.P. and T.A. performed PFGE analysis; T.T. designed the study. All authors contributed to manuscript preparation and approved the final version. The analysis of data presented here is a part of our routine effort endorsed by the Finnish Law, to prevent the spread of transmissible diseases. Therefore, a special permission from the Ethical Committee of the Eastern Finland region was not considered necessary. Verbal or written informed consent for the study was obtained from the patients.

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Travel-associated Illness Trends and Clusters, 2000–2010

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Longitudinal data examining travel-associated illness patterns are lacking. To address this need and determine trends and clusters in travel-related illness, we examined data for 2000–2010, prospectively collected for 42,223 ill travelers by 18 GeoSentinel sites. The most common destinations from which ill travelers returned were sub-Saharan Africa (26%), Southeast Asia (17%), south-central Asia (15%), and South America (10%). The proportion who traveled for tourism decreased significantly, and the proportion who traveled to visit friends and relatives increased. Among travelers returning from malaria-endemic regions, the proportionate morbidity (PM) for malaria decreased; in contrast, the PM trends for enteric fever and dengue (excluding a 2002 peak) increased. Case clustering was detected for malaria (Africa 2000, 2007), dengue (Thailand 2002, India 2003), and enteric fever (Nepal 2009). This multisite longitudinal analysis highlights the utility of sentinel surveillance of travelers for contributing information on disease activity trends and an evidence base for travel medicine recommendations.

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International travel is markedly increasing. In 2010, an estimated 940 million tourists arrived at international destinations, more than twice the 435 million in 1990 (*1*). Trips to developing regions have risen from 31% of all travel in 1990 to 47% in 2010, and trips to the Asia–Pacific region, Africa, and the Middle East have doubled in the past decade (*1*). Reasons for travel have also changed; from 1990 to 2010, trips for tourism decreased from 56% to 51%, and trips by those with ties to the destination country (travel for the purpose of visiting friends and relatives) increased from 20% to 27% (*1*,*2*).

More than half of international travelers to developing countries become ill during their trip, and ≈8% seek medical care for a travel-associated illness either during or after travel (*3*). Changes in travelers' illnesses over time would be expected to reflect changing patterns of global travel destinations, changes in local disease epidemiology

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in regions visited, and/or availability of preventive measures such as vaccination and chemoprophylaxis. To examine illness trends and clusters among travelers, we analyzed multisite longitudinal data collected by GeoSentinel sites during 2000–2010.

Materials and Methods

The GeoSentinel Surveillance Network (www.geosentinel.org) is a global network of specialized travel and tropical medicine providers. It was established through the International Society of Travel Medicine and the US Centers for Disease Control and Prevention (CDC) (*4*). Since 1997, data have been collected on illnesses imported across international borders by travelers and immigrants. GeoSentinel sites have been added progressively over time; currently, 54 clinics in 26 countries contribute data (Figure 1). Information is recorded for demographics, travel history, reason for travel, clinical signs and symptoms, and diagnosis. All sites use the best available reference diagnostic tests and base the identification of country (or region) of illness acquisition on itinerary, known endemicity patterns, and incubation periods. GeoSentinel sites enter their deidentified questionnaire-based information into a central SQL (structured query language) database. The GeoSentinel data-collection protocol was reviewed by the institutional review board officer at the CDC and was classified as public health surveillance, not as human subjects research requiring submission to institutional review boards.

The mix of patients and diagnoses reported by individual GeoSentinel sites varies according to site location and clinic type (hospital or outpatient). To examine trends over time, we included only sites that consistently reported posttravel data throughout the 11-year period of interest. From these sites we examined inpatient and outpatient data for trends in demographics, reason for travel, and proportionate morbidity (PM) for certain key diagnoses. Specific infections were included in analyses on the basis of clinical relevance plus sufficient case numbers (Table 1). PM is expressed as number of cases/1,000 ill travelers returned from the region(s) of interest. Where model fit of the PM variation over time was adequate (assessed statistically as the proportion of variance explained by year and considered adequately fitted if the coefficient of determination [R^2 statistic] was >50%), the rate of change in proportion was estimated by using linear regression with year as the independent variable and by using p value to assess the null hypothesis that there was no change over time. Statistical significance was set at $p<0.05$. Statistical procedures were performed by using Stata 10/IC for Windows (StataCorp LP, College Station, TX, USA).

Case clustering was assessed by using the scan statistic on georeferenced data. Cases were georeferenced to the centroid of the likely country of acquisition, and rate estimates were based on the total number of ill returned travelers from that country who visited GeoSentinel sites. Diseases examined–dengue, malaria, and enteric fever–were chosen because of clinical importance and relative frequency. The scan statistic uses a Poisson model to estimate the number of cases of each disease relative to the population returning from a given area. To encompass changes in season, the temporal window was set to 3 months. The spatial window was set to a 1,000-km radius. The scan statistic was calculated by using SatScan 9.1.1 (Kulldorff M; Information Management Services Inc., Boston, MA, USA), and significance was assessed by using 1,000 Monte Carlo simulations.

Results

Of 54 current GeoSentinel sites, 18 reported consistently during 2000–2010: 12 sites in North America, 2 in Australasia, and 4 in Europe/Middle East. A total of 42,223 ill returned travelers were reported and included in this study. The annual increase in patient numbers reported over the 11 years was statistically significant (+5%/year, $p = 0.03$).

Figure 1. GeoSentinel regions.

Table 1. Major diagnoses for returning travelers visiting 18 GeoSentinel sites, 2000–2010*

Diagnosis	No. cases
Malaria	1,762
Giardiasis	1,296
Dengue fever	888
Campylobacteriosis	596
Cutaneous larva migrans	577
Rabies postexposure prophylaxis	349
Enteric fever†	262
Spotted fever rickettsiosis	220
Chikungunya	120
Acute hepatitis A	94
Confirmed influenza A/B	84

*Other diagnoses included nonspecific gastrointestinal or diarrheal syndromes (≈25% of all patients); nonspecific febrile illness or viral syndrome (≈10%); rash, itch, or skin infection (≈10%); respiratory syndrome (≈5%); and other infectious and noninfectious problems. †*Salmonella enterica* serovar Typhi, *S. enterica* ser. Paratyphi, or unspecified.

Reason for Travel

Tourists comprised 63% of ill returned travelers overall, but over the study period, this proportion decreased by 10% (p = 0.009). By contrast, the proportion of those who traveled to visit friends and relatives increased from 9.1% in 2000 to 16.3% in 2010 (p = 0.002). The proportion of ill returned business travelers and missionaries/volunteers remained unchanged (Figure 2, panel A); the proportion of patients who reported having received pretravel advice declined, but not significantly (–5%, p = 0.07).

Destinations

The most common destinations from which ill travelers returned were sub-Saharan Africa (26%), Southeast Asia (17%), south-central Asia (15%), and South America (10%) (Figure 2, panel B). Over the 11-year period, the proportion of those returning from south-central Asia increased significantly (+5%, p = 0.028).

Diseases

Malaria

The most common sites for acquiring malaria were sub-Saharan Africa (77%), Oceania (6%), and south-central and Southeast Asia (5% each). However, the PM for malaria was greater for ill travelers returning from Oceania (average 248 malaria cases/1,000 ill travelers from the region) than for those returning from sub-Saharan Africa (average 135 cases/1,000 travelers). In 2000, the overall PM for malaria was 68 cases/1,000 ill travelers, and during 2000–2010, the rate decreased by an average of 30/1,000 (p = 0.002) (Figure 3, panel A). Despite overall increasing visits to GeoSentinel sites during the study period, absolute case numbers for malaria decreased (211 malaria cases reported in 2000; 151 in 2006, 124 in 2008, 189 in 2010) (Figure 3, panel B). The PM (and absolute case numbers) rose marginally during 2009–2010, compared with 2008, but did not negate the overall decreasing trend over the study period, which was most marked among ill travelers returning from Oceania (-204/1,000, p = 0.010), sub-Saharan Africa (–68/1,000, p = 0.003), and Southeast Asia (-31/1,000, p = 0.005).

PMs for malaria caused by *Plasmodium falciparum* and *P. vivax* decreased (-13/1,000, p = 0.012, and -13/1,000, p = 0.001, respectively). At a regional level, PMs for falciparum malaria decreased among ill travelers returning from sub-Saharan Africa (-39/1,000, p = 0.010), and PMs for vivax malaria decreased among those returning from Oceania (-169/1,000, p = 0.005), sub-Saharan Africa (-19/1,000,

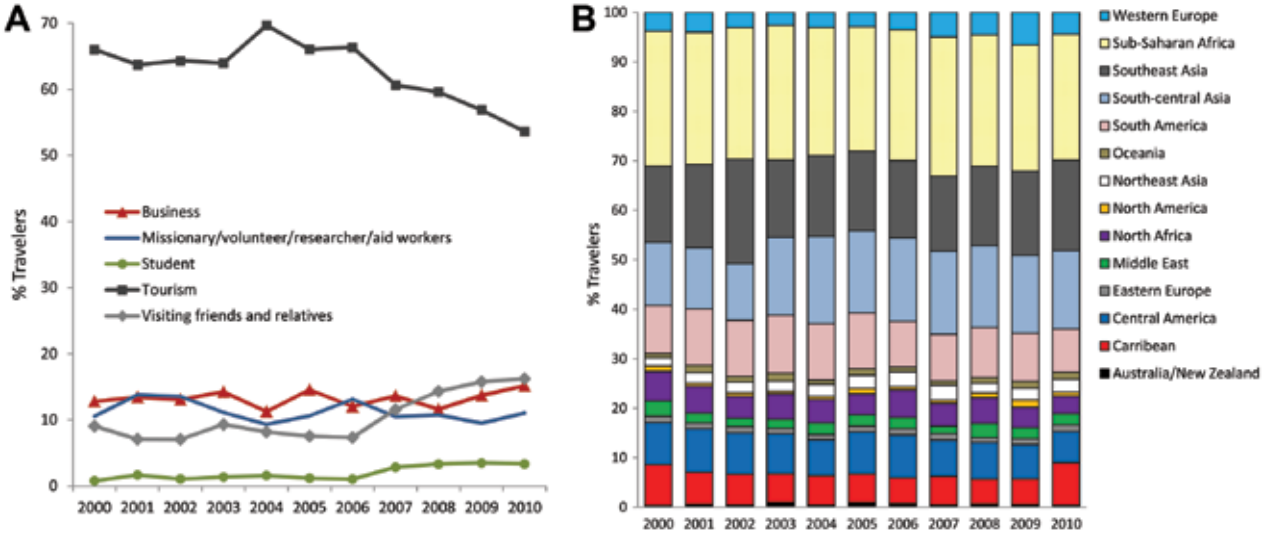

Figure 2. A) Reason for travel among 42,223 ill returned GeoSentinel patients, 2000–2010. Reason for travel missing for 188 (0.4%) patients. B) Destinations of travel among 42,223 ill returned GeoSentinel patients, 2000–2010. Region missing or unable to be determined (>1 region was visited) for 3,601 (8.5%) patients.

p < 0.001), and Southeast Asia (-24/1,000, p = 0.009). Decreasing PM trends for malaria were also small but significant among ill returning tourists (-37/1,000, p<0.001), travelers who visited friends and relatives (-103/1,000, p = 0.011), and business travelers (–43/1,000, p = 0.007).

Enteric Fever

For enteric fever (caused by *Salmonella enterica* serovar Typhi, *S. enterica* ser. Paratyphi, or unspecified), 67% of cases were imported from south-central Asia and 10% from each of Southeast Asia and sub-Saharan Africa (Figure 4). The PM for enteric fever increased over the 11 years (+10/1,000, p = 0.013). Exclusion of the 2009 cluster (Table 2) did not negate the overall significant trend. Regional trends could not be assessed because of considerable year-to-year variation in PM by region. Tourism accounted for 55% of cases and travel to visit friends and relatives for 27%.

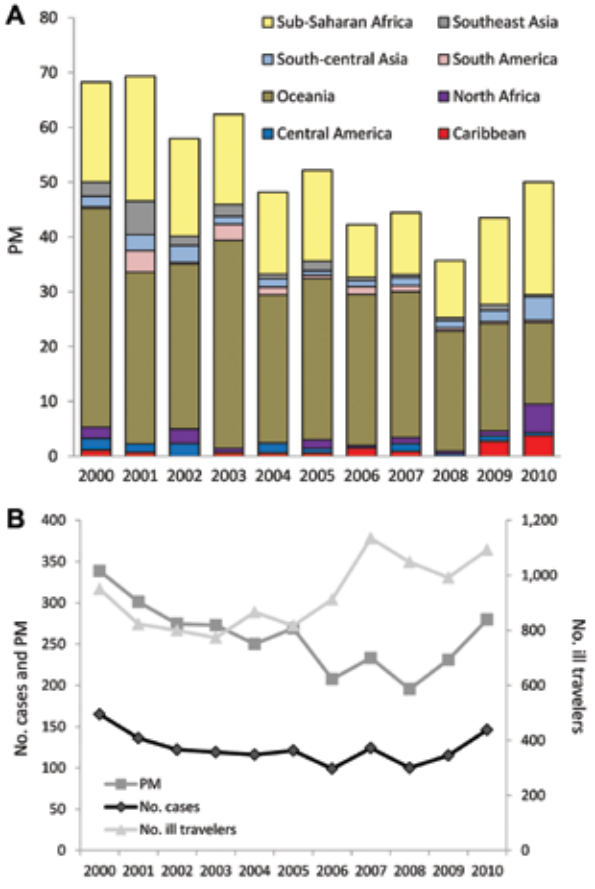

Figure 3. A) Proportionate morbidity (PM) for malaria (no. malaria cases/1,000 ill returned GeoSentinel patients) by region, 2000–2010. B) Absolute case numbers and proportionate morbidity for malaria (no. malaria cases/1,000 ill returned GeoSentinel patients) after travel to sub-Saharan Africa, 2000–2010. There were 1,363 total reported cases of malaria after travel to sub-Saharan Africa among the 18 GeoSentinel sites.

Dengue

With regard to dengue, 50% of patients had visited Southeast Asia; 17% south-central Asia; 9%–10% each Central America, South America, or the Caribbean; and 5% sub-Saharan Africa. There was considerable year-to-year PM variation not accounted for by a linear trend, largely because of a clear peak in 2002 (associated with an outbreak in Thailand) (*6*) (Figure 5). Excluding this peak, the underlying dengue PM (+26/1,000, p = 0.006) and case numbers (26 in 2000, 169 in 2010) increased significantly, especially among ill travelers returning from Southeast Asia (+71/1,000, p = 0.004) and sub-Saharan Africa (+8/1,000, p = 0.005).

Other

For chikungunya, the PM for total infections increased, as did the PM for cases acquired in Southeast Asia and south-central Asia. However, variation in PM was not well accounted for by a linear trend either overall or by region (Figure 6, panel A).

The PM for confirmed influenza A or B also increased. Similar to chikungunya, the variation in PM was not well accounted for by a linear trend, mostly because of the high number of visits during the 2009 pandemic (Figure 6, panel B).

The PM for rabies postexposure prophylaxis (PEP) increased significantly (+153/1,000, p<0.001) (Figure 6, panel C), particularly among those returning from Southeast Asia (+49/1,000, p = 0.001). No significant trends were found for hepatitis A, campylobacteriosis, giardiasis, cutaneous larva migrans, or spotted fever rickettsiosis.

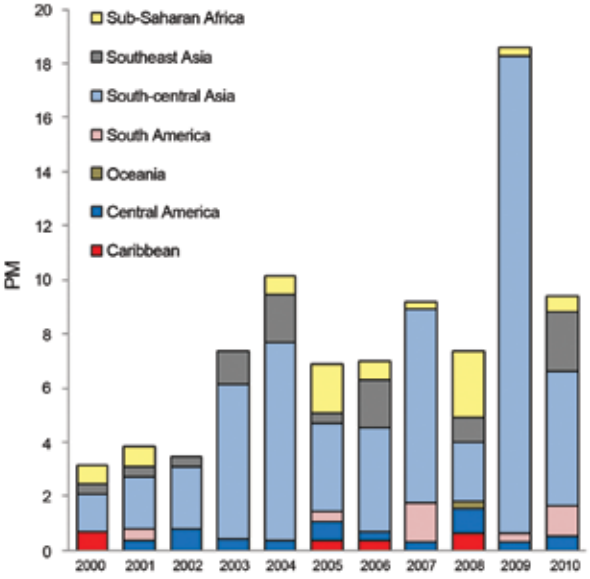

Figure 4. Proportionate morbidity (PM) for enteric fever (no. enteric fever cases/1,000 ill returned GeoSentinel patients) by region, 2000–2010.

Table 2. Main clusters detected among GeoSentinel patients, 2000–2010

Diagnosis, cluster	Location and dates of clusters	Cases/no. ill travelers (no. expected cases, p value)	Comments
Malaria			
Cluster A	Benin, Togo, Ghana, Burkina Faso, Nigeria, Cote d'Ivoire, 2007 mid Jul–mid Oct	44/185 (2.6, p<0.0001)	No definitive outbreaks discernible on ProMED-mail or in published literature corresponding to these clusters
Cluster B	Mauritania, Western Sahara, Mali, Senegal, 2000 mid Sep–mid Oct	19/53, (0.5, p<0.0001)	
Enteric fever	Nepal, 2009 Oct 5–20	24/40 (0.03, p<0.0001)	Associated with an outbreak of *Salmonella* paratyphi A among Israeli travelers to Nepal (*5*)
Dengue			
Cluster A	Thailand, 2002 Apr–Jul	44/257 (1.7, p<0.0001)	Reported in (*6*)
Cluster B	India, 2003 Sep–Nov	13/368 (0.9, p<0.0001)	

Cluster analyses were performed only for malaria, enteric fever, and dengue. Results are shown in Table 2.

Discussion

Sentinel surveillance of travelers is increasingly being recognized as an integral element for identifying emerging infections and disease outbreaks (*7*). However, the historical absence of systematic longitudinal data on travelers means that there are no studies on long-term disease trends among travelers and no data on whether traveler importation of illness mirrors regional disease trends in local populations. GeoSentinel surveillance has been performed continuously for >10 years and enables examination of longitudinal disease trends and clusters among returning ill travelers.

This 11-year analysis of ≈42,000 returned ill travelers identified several significant findings. Almost 60% had visited sub-Saharan Africa, Southeast Asia, or south-central Asia, but the largest regional fluctuation was in south-central Asia (+5%, p = 0.028). The proportions of ill tourists (-10%) and ill travelers who had visited friends and relatives (+9%) changed inversely. Although those who visit friends and relatives are at high risk for many travel-related health problems, pretravel advice and adequate precautions are often lacking (*8*,*9*).

Trends in morbidity rates for individual illnesses among travelers are influenced by many factors, including changes in disease incidence in regions visited, variations in uptake of preventive measures, and diagnostic factors. We report PM for specific illnesses, which is additionally influenced by changes in the number of travelers seen at GeoSentinel sites for other illnesses; therefore, interpretation of the longitudinal trends in PM requires caution. However, the proportion of the most common other illnesses seen was consistent (±3%) over the study years. Moreover, the lack of significant trends over time for some diseases examined, the decreasing trends for some illnesses, and the increasing trends for others suggest that these trends reflect real (albeit small) changes in the patterns and relative frequency of returned-traveler visits to specialist centers for these illnesses. In particular, the significant increase in proportion of ill travelers returning with enteric fever or dengue or seeking rabies PEP suggests that these conditions might have rising relevance for clinicians caring for ill returned travelers. For malaria, the average PM decreased significantly over the study period. Consistent regional trends were also seen, such as the high PMs for malaria among ill travelers returning from Oceania and sub-Saharan Africa, for enteric fever among those returning from south-central Asia, and for dengue among those returning from Southeast Asia (Figure 7). We also found significant clusters for malaria, enteric fever, and dengue.

Examination of simultaneous global changes in malaria epidemiology shows decreasing numbers of cases and deaths since 2000 (*10*,*11*). This decrease has been associated with increased funding for international malaria control, which has facilitated implementation of improved preventive and therapeutic interventions. Additionally, case numbers might have been overestimated, but they are now being rectified by increased use of rapid diagnostic tests (RDTs) and better case ascertainment.

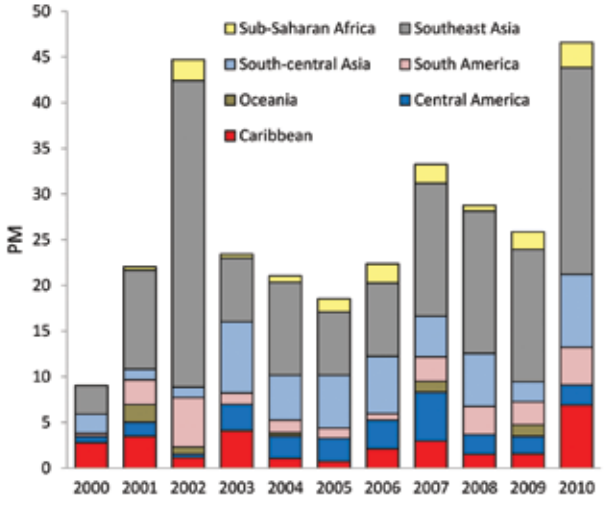

Figure 5. Proportionate morbidity (PM) for dengue (no. dengue cases/1,000 returned GeoSentinel patients) by region, 2000–2010.

One question is whether the observed overall decreasing trend in malaria PM is a travel medicine prevention success, a reflection of declining risk at destinations, a result of changes in diagnostics, or a combination of factors. Changes in diagnostic tests at GeoSentinel sites over the past decade, specifically more routine use of RDTs, are unlikely to have had much influence on malaria diagnosis at these specialized sites where experienced microscopists are available. Even if sensitivity of malaria diagnosis has increased with use of RDTs, this increase would not explain the observed decline in malaria PM. Because rates of imported malaria cases among travelers are influenced by use of personal protective measures and chemoprophylaxis, the decrease in PM might be partly attributable to better tolerated and more targeted prophylaxis (such as atovaquone/proguanil, available since 2000). However, the declining average PM trend over the study period (-28/1,000 ill travelers) is also consistent with reported global epidemiologic trends (*12*) resulting predominantly from decreased falciparum malaria cases. PMs for both falciparum and vivax malaria decreased significantly among ill persons returning after travel for tourism, visits to friends and relatives, and business.

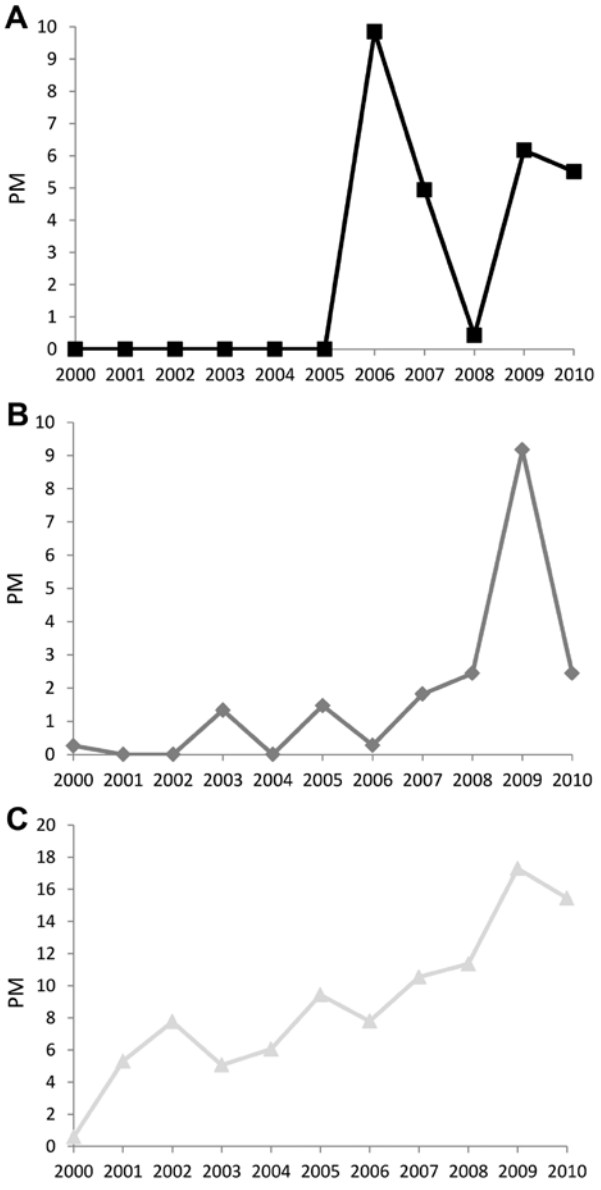

Figure 6. Proportionate morbidity (PM) (no. cases/1,000 returned GeoSentinel patients), 2000–2010. A) chikungunya, B) influenza, and C) rabies postexposure prophylaxis. Trends for chikungunya and influenza were not calculated because of substantial nonlinear year-to-year variation.

Since 2009, resurgence of malaria cases has been observed in several African countries, including Rwanda, São Tomé and Príncipe, Zambia, and Cape Verde (*10*). Our data also showed a slight increase in malaria PMs over the past 2 years (Figure 3); this finding is consistent with European and US data on imported malaria PMs for 2008–2010 (*13*,*14*) but in juxtaposition to the reported global decline in case numbers.

About three quarters of all malaria cases occur in Africa (*10*); among cases reported here, 77% were acquired in sub-Saharan Africa. Case clustering was detected in 2000 and 2007 among travelers with malaria returning from Africa, but a review of electronic reports and relevant literature revealed no definitive specific corresponding outbreaks.

S. enterica ser. Typhi and Paratyphi cause an estimated 20 million cases of enteric fever and 200,000–600,000 deaths annually in disease-endemic countries; cases have been increasing globally (*15*). Growing drug resistance is compounding the associated public health problem (*16*,*17*). In industrialized countries, the proportion of travel-related cases has risen (*17*,*18*), particularly *S. enterica* ser. Paratyphi cases, which are not prevented by current typhoid fever vaccines. Our results showing that two thirds of cases were among ill travelers returning from south-central Asia and that enteric fever PM is increasing (+10/1,000, $p = 0.013$) are consistent with global trends (*19*,*20*).

In October 2009, cluster analysis for enteric fever detected greater importation than expected among ill travelers returning from Nepal. This increase was associated with a large outbreak among travelers from Israel who were vaccinated for *S. enterica* ser. Typhi (Vi vaccine) but who contracted *S. enterica* ser. ParatyphiA infection in a restaurant in Pokhara, Nepal (*5*).

Among GeoSentinel patients with dengue, 50% acquired infection in Southeast Asia; the PM for dengue (adjusted for the 2002 outbreak) increased significantly. Worldwide each year, 50–100 million dengue infections occur (*21*), nearly 75% in Southeast Asia and the Western Pacific region (*22*). In the past 50 years, reported incidence has increased 30-fold; dengue has expanded into new countries and into urban settings (*22*,*23*) associated

Figure 7. Regional results for malaria, enteric fever, and dengue. For malaria, the top region for acquisition was sub-Saharan Africa (77%), and the region with the top average proportionate morbidity (248/1,000 ill travelers) and the greatest trend (-39/1,000, p = 0.01) Oceania. For enteric fever, the top region for acquisition was south-central Asia (67%); regional trends were not assessed. For dengue, the top region for acquisition (50%) and the highest average proportionate morbidity and trend (+70.5/1,000, p = 0.004) was Southeast Asia.

with population growth, urbanization, development of peri-urban slums, movement of virus by infected travelers, and improved diagnostic capabilities (*21*,*24*). The marked increase in cases in dengue-endemic regions is also reflected by data reporting infection among international travelers; prospective seroconversion studies estimate attack rates among travelers to the tropics to be 1.0%–6.7% (*25*–*28*). However, improved awareness of dengue and improved diagnostics, especially with PCR and nonstructural protein 1 antigen testing now being routinely available, might well underpin the observed trend in dengue diagnoses among ill returned travelers. Travelers have also been reported to serve as sentinels for dengue infection: GeoSentinel data showed that travel-related dengue reflected defined regional seasonality, and natural annual oscillations of cases among populations in dengue-endemic regions were also observed among travelers to these regions (*6*). In 2002, an increase in travel-related dengue activity among GeoSentinel patients returning from Thailand was noted before an outbreak was recognized by official Thailand surveillance data (*6*). Increased dengue cases among ill returned travelers from south-central Asia in 2003 were also evident before official surveillance data were available. Not surprisingly, cluster analyses detected these cases, but our results additionally represent long-term trends in dengue reflected by traveler surveillance data.

Our data suggest an increase in PM for chikungunya. Interest in chikungunya fever, long known to be endemic to tropical Africa and Asia, resurged in 2005–2006 when a large outbreak spread through the Indian Ocean islands and Asia–Pacific region (*29*,*30*). The continuing epidemic has affected populations in popular travel destinations; many imported cases among travelers have been reported (*31*).

The significant rise in the PM of persons seeking rabies PEP, particularly ill travelers returning from Southeast Asia, might result from an increased absolute risk for animal bites or scratches, or from increased high-risk exposures, high-risk activities, or awareness of rabies risk among travelers resulting in more visits for PEP. Globally, the number of human rabies cases and deaths has decreased markedly over the past 20 years (*32*,*33*), but in parts of Indonesia (e.g., Bali) and China, it has increased (*34*).

Influenza clearly peaked in 2009, coinciding with the influenza (H1N1) pandemic. Although the underlying trend was not formally examined, the observed increase in number of cases (Figure 6, panel B) might reflect a real rise in the proportion of travelers acquiring influenza or might reflect a lowered threshold for referral to specialized clinics and better access to confirmatory diagnostics during this period.

This study has limitations. The GeoSentinel Surveillance Network captures data only on ill persons who visit specialized clinics, and these data do not represent all international travelers. GeoSentinel data can therefore not be used to calculate absolute risk; PM calculations are performed instead. PM is a complex measure and changes over time either because of changes in numbers of reported cases of the disease of interest or because of significant changes in other diagnoses. Patterns of travel also change over time and are influenced by political, economic, and cultural events. Consequently, interpretation of results is complex, and changes in PM reflect changes in the recognized levels of the specific illnesses seen at specialized sites over time rather than changes in absolute risk for disease acquisition. Where relevant, comments regarding changes in numbers of cases have also been included to verify that changes in frequency of other diagnoses do not explain reported PM trends. Because the relative case mix of patients and diagnoses differs by GeoSentinel site, analyses need to account for changes in visits to each site over time. Subanalyses were performed to ensure that no single site was unduly driving overall trends (data not shown). Because disease acquisition is affected by numbers of travelers to

each destination, type and duration of travel, preventive measures implemented, and many other factors, traveler surveillance data would not be expected to precisely mirror trends in illness among host populations.

Despite these limitations, the annual changes in PM, although small, showed statistically significant trends that correlate with regional trends in disease for many diagnoses examined. In particular, PM changes for 3 major travel-related illnesses reflect global trends in disease epidemiology; trends for malaria decreased and trends for enteric fever and dengue increased. When case numbers were sufficient, significant regional trends could also be detected. We have also shown that an algorithm for detecting case clusters can be used on longitudinal traveler surveillance data. These findings highlight how sentinel surveillance of travelers provides an additional layer in surveillance efforts that can be used to inform the international community about disease activity trends in disease-endemic areas. Additionally, the relative contribution of diagnoses among returned ill travelers from different regions provides useful information for provision of health advice before and after travel.

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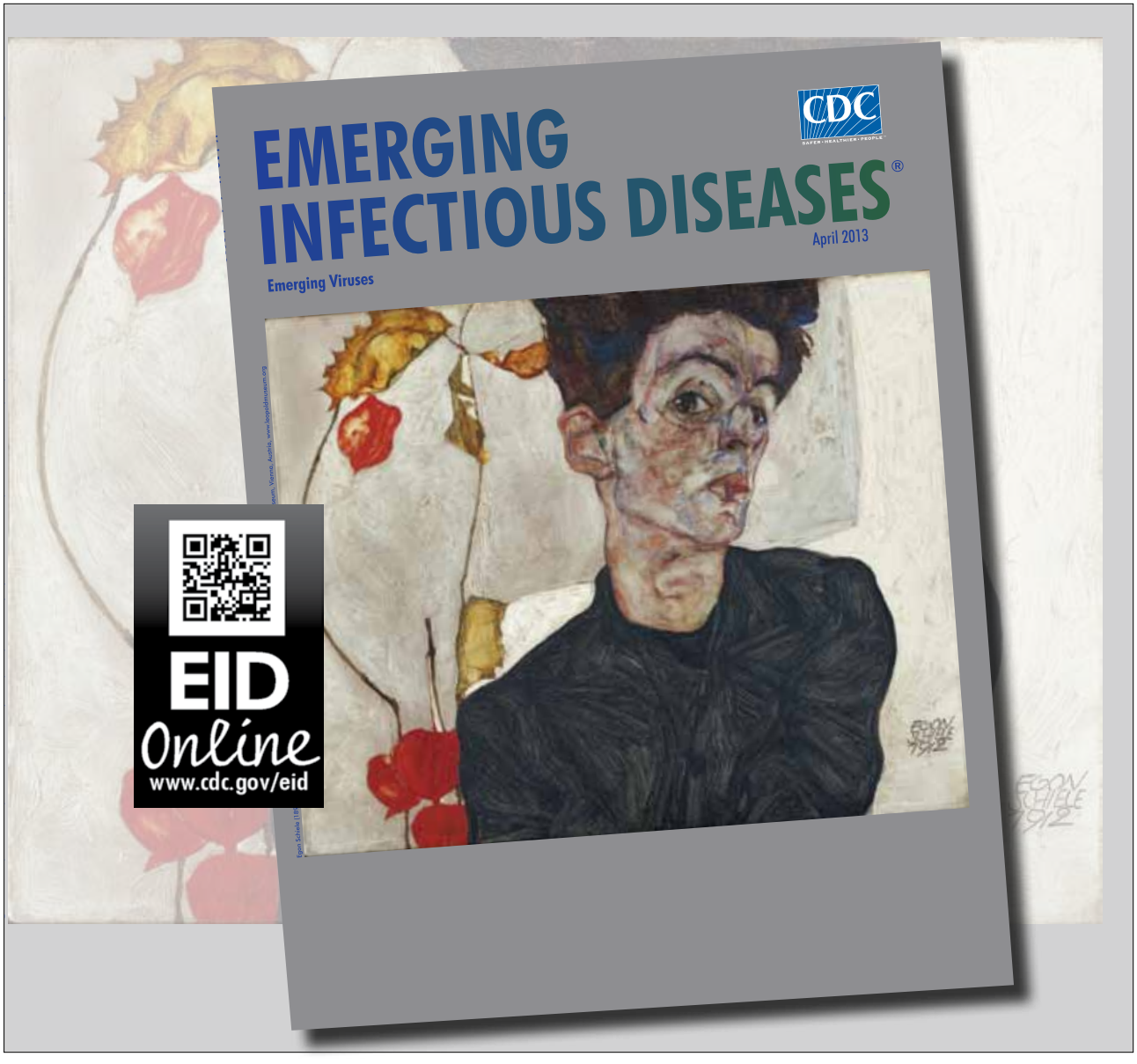

Quantifying Effect of Geographic Location on Epidemiology of *Plasmodium vivax* Malaria

Andrew A. Lover and Richard J. Coker

Recent autochthonous transmission of *Plasmodium vivax* malaria in previously malaria-free temperate regions has generated renewed interest in the epidemiology of this disease. Accurate estimates of the incubation period and time to relapse are required for effective malaria surveillance; however, this information is currently lacking. By using historical data from experimental human infections with diverse *P. vivax* strains, survival analysis models were used to obtain quantitative estimates of the incubation period and time to first relapse for *P. vivax* malaria in broad geographic regions. Results show that Eurasian strains from temperate regions have longer incubation periods, and Western Hemisphere strains from tropical and temperate regions have longer times to relapse compared with Eastern Hemisphere strains. The diversity in these estimates of key epidemiologic parameters for *P. vivax* supports the need for elucidating local epidemiology to inform clinical follow-up and to build an evidence base toward global elimination of malaria.

The malaria parasite *Plasmodium vivax*, which received limited research attention for a number of decades, has moved onto the global health agenda for 2 key reasons. First, compared with the *P. falciparum* parasite, which also causes malaria, the *P. vivax* parasite is more difficult to eliminate because it has a broader geographic range and, unlike *P. falciparum*, has dormant liver stages. Second, *P. vivax* malaria has reemerged in previously malaria-free temperate regions, including Greece, Corsica, the Korean Peninsula, central China, and Australia (*1*–*5*). Moreover, increasing evidence indicates that *P. vivax* infections can be severe and fatal (*6*,*7*).

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A large body of epidemiologic and clinical data supports the existence of discrete strains within each *Plasmodium* species. Much of the reliable data are from patients who were infected in temperate climates (study centers in the United States, United Kingdom, and Europe), and the parasites were from various locales. Tropical vivax strains generally cause a larger number of closely spaced relapses, and temperate strains have generally evolved to cause a long incubation period, enabling the survival of the parasite as dormant hypnozoites during colder months (*8*,*9*). Malariologists have long recognized that the incubation period for malaria varies by strain and geographic latitude (*10*).

Recent studies of temperate *P. vivax* strains in South Korea support these observations and suggest that chemoprophylaxis of infected patients may contribute to long latency (*11*,*12*). However, these observational studies could not estimate the time from infection to relapse or relapse periodicity because exact infection times were unknown. In a related primaquine dosing study, the relapse rate for 3 vivax strains was compared, but relapse time was not examined (*13*).

Numerous *P. vivax* classification schemes have been suggested, including temperate/tropical, temperate/subtropical/temperate, and northern/southern/Chesson-type. These schemes were suggested on the basis of observed clinical characteristics, but quantitative data to support these distinctions are sparse (*14*–*16*). Recent molecular and entomologic data suggests that *P. vivax* may consist of 2 subspecies, 1 in the Old World/Eastern Hemisphere (*P. vivax vivax)* and the other in the Americas (*P. vivax collins*) (*17*); however, research with other isolates has not confirmed these results (*18*). These 2 strains/subspecies show remarkable differences in their infectivity to different *Anopheles* spp. mosquitoes; however, the effect of these differences on the epidemiology of malaria in humans has not been demonstrated. In addition, at least 2 other subspecies, *P. vivax hibernans* and *P. vivax*

multinucleatum, produce disease that is similar to currently circulating strains from the Korean Peninsula (*19*,*20*).

A large body of historical data exists from the deliberate laboratory infection of 2 populations: 1) patients receiving malariotherapy (intentionally induced malaria) for neurosyphilis and related disorders; and 2) healthy prisoners who participated in malaria drug testing trials. We used the experimental infection data from these controlled settings to explore and quantify the relationship between *P. vivax* parasite origin and characteristics of malaria caused by these subpopulations.

Materials and Methods

Data Sources and Selection Criteria

Search Strategy

We performed a comprehensive literature search of MedLine and Google Scholar in English, searching for ("vivax" OR "benign tertian") AND ("induced" OR "human" OR "experimental"). Limited searches were performed in Dutch, German, and French. The citations in these initial papers were examined, resulting in the identification of a large number of non-indexed papers.

Data Inclusion Criteria

We included data for experimentally infected persons who 1) were malaria naive before the experimental infection, 2) had defined inoculation dates (infection by mosquito only; persons with infections from sporozoite injection or blood transfer were excluded), 3) had received only well-documented (or explicit) treatment of symptoms, 4) were protected against reinfection, and 5) were infected with traceable strains that had defined origins. Studies that measured prepatent period (i.e., the time to first appearance of blood-stage parasites, seen by microscopy) were excluded. Data for patients with incubation periods >50 days were excluded from the analysis because only 7 such cases were found in well-defined studies. For the infection relapse study, we included data only from studies that had unambiguous follow-up periods. Available covariates were extracted from these studies, and the individual records were digitized by using Plot Digitizer (http://plotdigitizer.sourceforge.net), as needed, to create individual case-patient records.

Our incubation period analyses included data for 453 patients (infected with a total of 11 strains) from 19 studies (Table 1); data for 6 of the patients were excluded because of highly outlying covariate patterns (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/12-1674-Techapp1.pdf). The first relapse analyses included data for 320 patients (infected with a total of 18 strains) from 15 studies (Table 1); data for 4 of the patients were excluded because of highly outlying covariate patterns (online Technical Appendix). Details of the parasite strains are shown in Table 2. Age and sex were not recorded for most patients who had neurosyphilis. All prison volunteers were White men. Several sources had interval censoring; that is, infections were reported as occurring within 1 month or 16 weeks of the mosquito bite. To facilitate comparisons between studies that reported relapse times by integer weeks, relapse dates reported as exact days were analyzed as the next full week from infection. The final relapse event in these data occurred 65 weeks after initial infection; longer follow-up times were right-censored at 78 weeks from initial infection. Latitude of parasite isolation was determined from the site of isolation in the original reports and coded as a binary variable (dividing at ±23.5° South/North latitude) for determining tropical and temperate *P. vivax* parasites. In our analyses, New World strains are those from the Americas, and Old World strains are those from Eurasia, Africa, and the Pacific region, as suggested by Li et al. (*17*).

Table 1. Infection data recorded for 677 case-patients in historical studies of *Plasmodium vivax* infection, circa 1920–1980*

Infection data	No. (%) patients
Neurosyphilis/neurologic treatment patient	433 (64.0)
Recorded data	
Exact incubation period only	356 (52.6)
Time to relapse only	217 (32.1)
Exact incubation and time to relapse	104 (15.4)
Infected with	
Tropical strains	275 (40.6)
Temperate strains	402 (59.4)
New World strains	181 (26.7)
Old World strains	496 (73.3)

*The study population was followed for a mean of 82.5 weeks (SD 31.7, range 2.0–173.0).

Statistical Analyses

Kaplan-Meir analysis was used to examine the unadjusted relationship between parasite origin and event times; differences were assessed by using a log-rank test. Multivariate models were used to overcome limitations of Kaplan-Meier analyses by allowing for adjustment for the effect of malariotherapy and by producing hazard ratios (HRs) to gauge the strength of association. The standard Cox proportional hazards model cannot provide confidence intervals for predicted survival times; therefore, the more complex, flexible parametric Royston-Parmar models were used to provide covariate-adjusted HRs and covariate-adjusted median survival times for parasite subpopulations (*21*). These models extend Cox methods by adding parameters that model the underlying hazard of a disease event, which allows for more comprehensive predictions. To provide estimates that are relevant to natural malaria infections in humans, the predicted survival times from this analysis were made for a population not receiving treatment for neurologic symptoms.

Table 2. *Plasmodium vivax* strains included in a study quantifying the effect of geographic location on the epidemiology of *Plasmodium vivax* malaria

Strain	Place of origin, date	No. case-patients	Malariotherapy	% Case-patients given malariotherapy	Region of origin
Chesson	Papua New Guinea, circa 1944	145	No		Tropical
Hlebnikovo	Moscow Oblast, 1948	19	Yes	100.0	Temperate
Holland	Netherlands, circa 1928	52	Yes	100.0	Temperate
Korea	North Korea, 1953	21	Yes	100.0	Temperate
Leninabad	Tajikistan, 1950	33	Yes	100.0	Temperate
Madagascar	Madagascar, 1925	83	Yes	100.0	Tropical
McCoy	Florida, USA, 1931	70	Yes	100.0	Temperate
Moscow	Moscow, 1950	55	Yes	100.0	Temperate
NICA (Nicaragua)	Nicaragua, circa 1970	6	No		Tropical
Nahicevan	Azerbaijan, 1937	5	Yes	100.0	Temperate
Naro-Fominsk	Moscow Oblast, 1946	21	Yes	100.0	Temperate
Panama	Panama, circa 1970	10	No		Tropical
Rjazan	Ryazan, Russia circa 1945	21	Yes	100.0	Temperate
St. Elizabeth	Southern USA, circa 1925	73	Yes	34.2	Temperate
Salvador I	El Salvador, circa 1970	11	No		Tropical
Salvador II	El Salvador, circa 1970	11	No		Tropical
South Vietnam	Southern Vietnam, circa 1972	5	No		Tropical
Vietnam (North)	Northern Vietnam, 1954	4	Yes	100.0	Tropical
Volgograd	Volgograd, Russia, 1945	24	Yes	100.0	Temperate
West Pakistan	Pakistan, 1968	5	No		Temperate
P. vivax multinucleatum	Central China, 1965	3	No		Temperate
Total		677		64.0	

The incubation period and time-to-relapse models include the geographic region and neurologic treatment status as covariates for survival time; for the time-to-relapse models, geographic region was modeled as a time-varying covariate due to proportional hazard violations (the effect of regions is allowed to vary through follow-up time). Both models also incorporate adjustments for intragroup correlation caused by the clustering of effects among persons infected with the same parasite strain.

We used Stata 12.1 (StataCorp, College Station, TX, USA) to perform statistical analyses; all tests were 2-tailed. Detailed methods are in the online Technical Appendix.

Results

Incubation Period

The Kaplan-Meier plot of 447 case-patients who were included in the incubation period study shows a wide separation of Old World/New World groups by tropical/temperate region (Figure 1, panel A) and latitude/Hemisphere (Figure 1, panel C). The separations are statistically discernible by tropical/temperate region (log-rank test for equality $\chi^2 = 127.9$, 1 df, $p<0.0001$) and by latitude/hemisphere (log-rank test for equality $\chi^2 = 204.6$, 3 df, $p<0.0001$).

The unadjusted median incubation period for malaria caused by the combined tropical strains was 12 days (95% CI 12–12); that for the combined temperate strains was 15 days (95% CI 14–16). When stratified by Old World/New World, the tropical strains remain essentially unchanged, but a large separation occurred in the temperate strains: median survival was 14 days (95% CI 14–15) for New World temperate strains and 20 days (95% CI 19–21) for Old World temperate strains.

In the full multivariate model, after adjustment for neurologic treatment status, the median parametric survival estimate for the entire population was 13.6 days (95% CI 12.4–14.7) (Figure 1, panel E). The 95th percentile for the incubation period was 17.8 days (95% CI 16.6–18.9). Adjusted HRs differed between all regional categories, except for New World tropical/temperate categories, which did not achieve significance ($p = 0.30$). Predicted median and 95th percentile survival times are shown in Figure 2. Predicted median survival times were not different within the confidence intervals, except for Old World temperate strains (20.1 days [95% CI 17.8–22.5]); the 95th percentiles for temperate strains were significantly longer than those for tropical strains. HRs were 16.8 (95% CI 7.7–36.9) for the Old World, tropical region; 10.8 (95% CI 4.6–25.2) for the New World, tropical region; 7.3 (95% CI 3.8–14.0) for the New World, temperate region—all relative to the Old World, temperate region (reference). Persons infected with Old World tropical strains had a 16.8 (95% CI 7.7–36.9) times higher risk of clinical infection than did those with Old World temperate strains at each time point, leading to a shorter incubation period.

Time to First Malaria Relapse

In all studies, the time to first relapse was measured from the reported primary infection. We used Kaplan-Meir survival curves to compare relapse times for the 316 persons included in these analyses and found large, statistically significant differences by tropical/temperate regions (log rank test for equality $\chi^2 = 56.2$, 1 df, $p<0.0001$) (Figure 1,

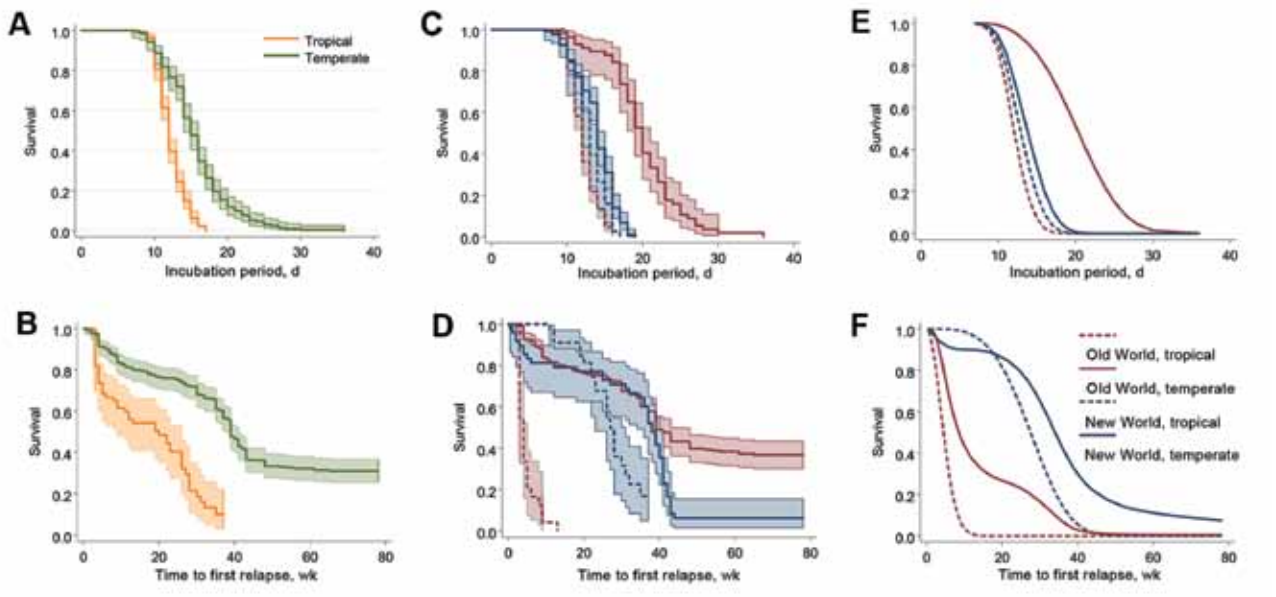

Figure 1. Various modeling estimates of incubation period and time to first relapse for *Plasmodium vivax* malaria in a study quantifying the effect of geographic location on the epidemiology of the infection. A) Kaplan-Meier estimates for incubation period, temperate/tropical strains. B) Kaplan-Meier estimates for time to first relapse, temperate/tropical strains (key in panel A). C) Kaplan-Meier estimates for incubation period, by region (key in panel F). D) Kaplan-Meier estimates for time to first relapse, by region (key in panel F). E) Flexible parametric survival model, incubation period projected for neurologic treatment–free populations, by region (key in panel F). F) Flexible parametric survival model, time to first relapse projected for neurologic treatment–free populations, by region.

panel B) and by latitude/hemisphere (log rank test for equality $\chi^2 = 198.9$, 3 df, $p<0.0001$) (Figure 1, panel D). Figure 1, panel D, shows that infections caused by the 2 Old World categories show shorter times to relapse compared with infections from New World parasites. In the full multivariate model (adjusted for neurologic treatment status), we found a distinct separation between the hemispheres (Figure 1, panel F); for the total population, we estimated the median time to relapse was 29.2 weeks (95% CI 25.0–33.4) and the 95th percentile was 61.4 weeks (95% CI 35.7–87.1).

However, these aggregate values obscure substantial heterogeneity in time to relapse. Median relapse times for malaria caused by Old World parasites (tropical, 4.5 weeks [95% CI 3.6–5.4]; temperate, 8.5 weeks [95% CI 6.8–10.3]) were shorter than those for malaria caused by New World parasites (tropical, 27.5 weeks [95% CI 21.6–33.5]; temperate, 34.0 weeks [95% CI 32.0–36.0]). In addition, in both hemispheres, median relapse times for infections caused by tropical strains were shorter than those for infections caused by corresponding temperate strains, although this difference was not significant in the New World (Figure 3). The 95th percentile relapse times for the strain categories follow: Old World tropical, 9.5 weeks (95% CI 5.4–13.5); New World tropical, 40.3 weeks (95% CI 34.4–46.3); Old World temperate, 30.9 weeks (95% CI 19.9–41.9); and New World temperate, 97.7 weeks (95% CI 97.6–97.8). The HRs from the survival models (adjusted for neurologic treatment) follow: Old World tropical, 39.6 (95% CI 9.2–171.0; $p<0.001$); New World tropical, 0.93 (95% CI 0.36–2.41; $p = 0.89$); Old World temperate, 3.1 (95% CI 2.2–4.6; $p<0.001$)–all relative to New World temperate (reference).

Distribution of Relapses

The total number of relapses was compared by latitude and hemisphere (online Technical Appendix). An interval of 48 weeks was chosen to ensure equivalent follow-up periods between the regions (1-way analysis of variance *F*-test, follow-up time by region [$p = 0.37$]). To test the equality of distributions, we use the Kolmogorov-Smirnov test and found significant differences between tropical and temperate strains ($p<0.001$) and Old World and New World strains ($p = 0.001$). These differences remained significant when stratified by zones of latitude: temperate strains, Old World versus New World ($p = 0.001$); and tropical strains, Old World versus New World ($p<0.001$).

Discussion

Malariologists have made a large number of observations concerning the geography-related epidemiology of *P. vivax* malaria (*22,23*). Malariotherapy treatments initially used a range of local strains from the United Kingdom, the Netherlands, and the United States, but these were quickly replaced with the Madagascar strain

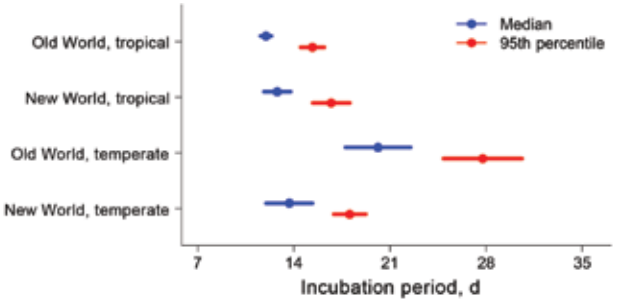

Figure 2. Length of incubation for *Plasmodium vivax* malaria infection, as determined by using flexible parametric survival models adjusted for neurologic treatment status, in a study quantifying the effect of geographic location on the epidemiology of the infection.

and others, which exhibited shorter incubation periods and more reliably produced infections (*24*). Estimates for the incubation period of *P. vivax* malaria vary: mean of 13.9 days (SD 3.7); 14 ± 3 days after the mosquito bite; 12–17 days (mean 15); and 8–17 days (*25*–*28*). A modern quantitative analysis of data for malariotherapy with a single strain (Madagascar) provided estimates of ≈10.3–16.9 days for the prepatent period (reported, in the study, to be generally 3 days longer than the incubation period) (*29*). After adjusting for neurologic treatment status, the estimated median from the parametric models for the population in our study is 13.6 days (95% CI 12.5–14.7), and the incubation period 95th percentile was 17.8 days (95% CI 16.6–18.9). The minimal differences between the Kaplan-Meier estimates and those from the multivariate models for incubation period strongly support earlier opinions that malariotherapy data are applicable to natural infections (*30*).

A range of strain-specific observations has also been reported for malaria relapses (*27*). Compared with persons with malaria caused by temperate strains, persons with malaria caused by tropical strains relapse more and have shorter relapses intervals (17–45 days), and a higher proportion have >2 relapses (*26*). It has been estimated that malaria caused by tropical strains relapses every 3–4 weeks, whereas malaria caused by temperate strains has longer, more variable periods between relapses (*31*).

Our finding suggests that these prior estimates for relapse intervals were based primarily on infections with Old World tropical strains. Our time-to-relapse estimates for other regions are considerably longer than estimates from earlier studies, with the exception of a study from El Salvador, which reported a median relapse interval of 28 weeks (*32*). The arithmetic median interval for malaria caused by all tropical strains (including only exact, non–interval censored times) was 20.0 weeks (95% CI 11.9–28.1). Results of the unadjusted Kaplan-Meier and parametric models, adjusted for neurologic treatment status in the full dataset, also indicate much longer median times to relapse: 20 weeks (95% CI 9–26) and 10.5 weeks (95% CI 3.7–17.4), respectively. Persons infected with Old World temperate strains have 39 (95% CI 9.2–171.0) times higher risk of relapse at each time point relative to those infected with New World temperate strains. Figure 1, panels D and F, suggests that the interaction between neurologic treatment and infection with different parasite strains has a substantial effect on the course of relapse; therefore, unadjusted relapse times from malariotherapy studies should be interpreted with caution.

In the data we studied, the number of relapses recorded within 48 weeks (for equivalent follow-up) is consistent with prior estimates: median of 2.6 relapses (95% CI 1.9–3.3; range 0–9) and of 0.68 relapses (95% CI 0.55–0.80; range 0–6) for case-patients with malaria caused by tropical and temperate strains, respectively. The data also showed that 68.5% (95% CI 64.9%–71.8%) of case-patients had relapses; this finding is broadly consistent with the previous finding that ≈60% of untreated cases relapse (*27*).

The general agreement of the number of relapses and incubation period among the population we studied with prior estimates suggests that the patients in our study do not represent a population substantially different from those with naturally acquired infections. However, the aggregate cohort values obscure large regional differences in epidemiology.

The effect of the geographic location of malaria parasites on the epidemiology of malaria has been long recognized: conspicuous differences in incubation period and disease latency have been broadly correlated with climatic zones (*27*). However, there have also been persistent difficulties in classifying these patterns; 2 different types of temperate strains (North American [St. Elizabeth] and European [Netherlands]) as well as tropical strains have been suggested (*14*). Inconsistencies have also been noted. For example, the tropical *P. vivax* strains in Central America show anomalous temperate zone epidemiology, leading to a suggestion that temperature alone might be an insufficient predictor of regional epidemiology (*33*). These conflicting observations are consistent with the results from this study (Figure 1, panel F), in which the most noticeable feature is that both categories of New World parasites caused malaria with substantially longer times to relapse, compared with malaria caused by Old World parasites.

Our findings suggest that the epidemiology of *P. vivax* infection has been occluded by inherent differences between parasite subpopulations and that hemisphere and latitude are strong drivers of the clinical manifestations and epidemiology of malaria. These findings strongly suggest that current paradigms for *P. vivax* clinical follow-up and surveillance may be based on erroneous assumptions. Malaria should not be discounted as a diagnosis even in the presence of long incubation periods, and the geographic

origin of the parasite has critical effects on clinical features and should not be ignored in case histories.

Our results show that the mean incubation period for malaria caused by *P. vivax* strains from Eurasian temperate zones is statistically and clinically significantly longer than generally considered. This, plus the inherently longer extrinsic incubation period for malaria caused by Old World temperate strains, suggests that an active surveillance period of 31 days after potential exposure is the minimum necessary to capture the 95th percentile of new cases. However, this surveillance interval is balanced by a shorter median time to relapse for Old World temperate strains relative to all New World strains.

These 3 sets of independent measures (i.e., incubation period, time to first relapse, and distribution of the total patient relapses) across the entire course of illness suggest that *P. vivax* should not be considered a single parasite but is, in fact, several discrete and clinically distinct populations with unique and measurable characteristics. These data, plus previous entomologic and molecular evidence, support the delineation of subspecies within the range of the parasite: *P. vivax vivax* in the Eastern Hemisphere and *P. vivax collins* in the Western Hemisphere. This conclusion is supported by a recently published phylogenetic analysis of global *P. vivax* strains, which shows high diversity and clustering of isolates by hemisphere (*34*).

The origin of the infecting parasite affects the prophylaxis and treatment of malaria. Infections caused by strains from Korean Peninsula respond to standard doses of primaquine, but even higher doses did not fully suppress strains from New Guinea (Chesson), and infections caused by the Chesson strain required twice the dose of quinine relative to infections by a North American strain (McCoy) (*16*). Another related study also found large regional differences: infections with Thai strains were more likely to relapse and required higher primaquine dosing relative to infections from India or Brazil (*13*). The effect of malaria parasite population differences should be considered in the planning and analysis of interventional trials and in potential vaccine trials.

A recent analysis of malaria imported into the United States and Israel found that a large proportion of the case-patients exhibited long periods of latency: of 721 *P. vivax* case-patients with insufficient/nonexistent antimalarial drug prophylaxis, 46.5% (95% CI 42.8%–50.2%) had an incubation period >2 months, compared with 80.0% (95% CI 77.0%–82.8%) of case-patients with sufficient prophylaxis [authors' calculations, from (*35*)]. However, no information was provided about the geographic source of these parasites, and the date of exposure is assumed to be the end of the travel period, making exact calculation of the incubation period impossible. The results from our study are broadly consistent with these values:

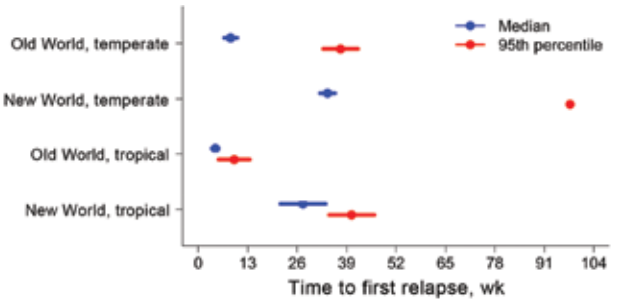

Figure 3. Predicted number of weeks from primary *Plasmodium vivax* malaria infection to first relapse, as determined by using flexible parametric survival models adjusted for neurologic treatment status, in a study quantifying the effect of geographic location on the epidemiology of the infection.

31.3% (95% CI 26.2%–36.6%) of case-patients in the time-to-relapse study had an incubation period >8 weeks. The comparability of these antimalarial drug–free populations suggests long-term stability in the global malaria parasite populations and also supports the relevance of these historical challenge studies for modern surveillance programs. The potential for prolonged incubation due to prophylaxis suggests that the estimates from our analysis should be considered as minimum values for travelers returning from vivax-endemic areas who had sufficient malaria prophylaxis.

Control and elimination programs for *P. vivax* should be reconsidered in light of these findings. Two major stumbling blocks identified during the First Global Malaria Eradication Campaign (1955–1972) were 1) the assumption that control methods could be universally applied and 2) burnout among program staff and funding agencies, resulting from continued surveillance at increasingly lower levels of infection (*36*). Addressing these issues in the current malaria elimination campaign will require detailed elucidation of the differences in pharmacodynamics among current parasite subpopulations and locale-specific malaria epidemiology, including estimation of incubation periods and the time to relapse to maximize surveillance efficiency.

Our results suggest that considerable complexity among *P. vivax* populations has been obscured by data aggregation; however, these divisions appear along defined geographic gradients. The long time interval of these studies (1920s–1980s) implies relatively stable parasite populations; however, the effect of greatly increased airplane travel, migration, and population-level antimalarial drug pressure should be explored.

The existence of subpopulations of *P. vivax* parasites along the division between the Eastern and Western Hemispheres enables conflicting historical and epidemiologic data to be formulated into a consistent and coherent picture, especially with the incorporation of phylogenetic

approaches. In addition, parasite origin should be considered in drug prophylaxis and treatment, and the epidemiologic differences in disease caused by *P. vivax* subpopulations should be more fully elucidated.

> Local malaria problems must be solved largely on the basis of local data. It is rarely safe to assume that the variables in one area will behave in the same way as they do in another area, however closely the two regions may seem to resemble each other in topography and climate. Large sums of money have been wasted in attempted malaria control when malariologists have forgotten this fundamental fact. (Paul F. Russell, 1946 [*37*]).

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Mutation in Spike Protein Cleavage Site and Pathogenesis of Feline Coronavirus

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Feline coronaviruses (FCoV) exist as 2 biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). FECV causes subclinical infections; FIPV causes feline infectious peritonitis (FIP), a systemic and fatal disease. It is thought that mutations in FECV enable infection of macrophages, causing FIP. However, the molecular basis for this biotype switch is unknown. We examined a furin cleavage site in the region between receptor-binding (S1) and fusion (S2) domains of the spike of serotype 1 FCoV. FECV sequences were compared with FIPV sequences. All FECVs had a conserved furin cleavage motif. For FIPV, there was a correlation with the disease and ≥1 substitution in the S1/S2 motif. Fluorogenic peptide assays confirmed that the substitutions modulate furin cleavage. We document a functionally relevant S1/S2 mutation that arises when FIP develops in a cat. These insights into FIP pathogenesis may be useful in development of diagnostic, prevention, and treatment measures against coronaviruses.

Feline infectious peritonitis (FIP) is a fatal infection that affects domestic and wild members of the family Felidae and is caused by a feline coronavirus (FCoV) of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*, species *Alphacoronavirus*-1 (*1*). The FCoV genome is ≈29 kB and has 11 open reading frames encoding replicative, structural, and accessory proteins (*2*). Two serotypes have been identified. Serotype 1 FCoVs are highly prevalent clinically (*3*–*5*) but grow poorly in cell culture and are therefore underevaluated when compared with serotype 2 FCoVs, which are easily propagated in vitro but less prevalent.

Within each serotype, there are 2 biotypes, each causing distinct disease outcomes. Feline enteric coronavirus (FECV) of serotypes 1 and 2 infects enterocytes, causing mild and generally self-limiting infections. FECV spreads efficiently through the oral–fecal route, and chronically infected cats can shed infectious virus in feces for a year or longer (*6*,*7*). The second biotype found in both serotypes, FIP virus (FIPV) is found less frequently but causes FIP.

The current understanding is that FIPV arises during in vivo infection from a genetic mutation of FECV (*8*–*11*). A long-standing hypothesis is that FIP viruses arise from internal mutation of endemic FECVs (*12*), which is believed to occur in approximately 1%–5% of enteric infections, resulting in the ability of the virus to infect blood monocytes and tissue macrophages. The resulting productive infection of these cells, a hallmark of FIP, enables systemic spread and results in macrophage activation, with concomitant immune-mediated events leading to death. To date, the precise mutation or mutations that cause a shift in FCoV biotype have not been identified.

As with other RNA viruses, coronavirus replication is error-prone; the estimated mutation rate is $\approx 4 \times 10^{-4}$ nucleotide substitutions/site/year (*13*,*14*). It has been suggested that mutations in the 3c and 7b genes may be involved in the transition to FIPV (*1*,*12*,*15*). Because FCoV spike protein plays critical roles in receptor binding (S1) and fusion (S2), we focused on structural changes in this protein and potential role in altered cellular tropism. In particular, acquisition of macrophage tropism for a serotype 2 FCoV has previously been mapped to the spike gene (*16*), further suggesting that key mutations within spike protein may be important for the biotype switch.

The coronavirus spike protein is a class I fusion protein, which typically requires activation by cellular proteases. Mutation of the proteolytic cleavage site often has profound implications for disease progression (*17*,*18*). Until recently, FCoVs were thought to have uncleaved spike

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protein. However, a functional furin cleavage site has been identified in 2 serotype 1 FECVs, located at the shared boundary of the S1 and S2 subunits (*19*). Furin is a ubiquitous proprotein convertase enriched in the trans-Golgi network and is well-conserved among mammals (*20*). Furin cleaves a wide range of protein precursors into biologically active products at a consensus motif R-X-K/R-R, where R is the basic arginine residue, X is any residue, and K is the basic lysine residue (*21*).

In this article, we establish a novel approach to studying FIP that complements previous work. Instead of performing a mutation study based mainly on comparative genetic analysis (*15*,*22*–*24*), we focus on S1/S2, a functionally relevant site, and study variations between the biotypes and their functional effects. This rationale could provide a better means to uncover functionally important mutations that account for FIP.

We considered that mutations at the S1/S2 site could alter proteolytic cleavage and modify S fusogenic properties, leading to tropism expansion, systemic spread and, ultimately, FIP. We investigated genetic variations at the S1/S2 site of serotype 1 FECVs and compared these sequences to those present in viral RNA recovered from tissues of cats with FIP. Fluorogenic peptide cleavage assays were conducted to assess the effects of substitutions found in the S1/S2 site. We document a junction mutation at S1/S2 that arises during development of FIP. Our study has uncovered a molecular basis for FIP that has potential to lead to developments in diagnostics, prevention, and therapies.

Materials and Methods

FCoV Sequence Analysis

Clinical and demographic data are reported in online Technical Appendix Table 1 (wwwnc.cdc.gov/EID/article/19/7/12-1094-Techapp1.pdf). Fecal samples from asymptomatic infected domestic cats were solicited from shelters and veterinarians throughout the United States. RNA was extracted by using QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). FCoV primers that detect most circulating strains were used to screen all fecal samples (*25*). RNA extracted from FIPV-TN406 (Black) laboratory-adapted strain was used as a positive control.

We analyzed 22 FIPV-positive tissue samples (Veterinary Pathology Archives, Cornell University, Ithacan, NY, USA) from 11 cats with FIP. Diagnosis of FIP was based on the standard method of immunohistochemical evaluation by board-certified pathologists. Each sample was retrieved from formalin-fixed, paraffin-embedded tissue blocks from which sections were stained by using FIPV 3–70 antibody (Custom Monoclonals, Sacramento, CA, USA). Positively stained regions were thinly sectioned and RNA was extracted by using RecoverAll (Ambion, Foster City, CA, USA).

Fecal samples collected from FCoV-positive housemates, cats 234 and 304, were processed as previously described in this section. After the referring veterinarian made a diagnosis of FIP in cat 234, the owner elected to euthanize the animal. Fresh tissue was harvested and RNA extracted by using MagMAX Express (Life Technologies, Grand Island, NY, USA).

For all samples, 50 μL reverse transcription PCRs (RT-PCRs) were performed with One-Step RT-PCR (QIAGEN) by using gene-specific S primers, encompassing S1/S2. The PCR primers sequences are found in online Technical Appendix Table 2. PCR conditions were 30 min at 50°C, 15 min at 95°C, and 39 or 35 cycles of 1 min at 94°C, 1 min at 55°C, 1 or 1.5 min at 72°C, and 10 min at 72°C. PCR products were purified by using a QIAquick Gel Extraction Kit (QIAGEN). Sanger sequencing was performed at the Life Sciences Core Laboratories (Cornell University). Nucleotide archive accession numbers are shown in online Technical Appendix Table 4. DNA sequences were translated into protein sequences and alignments were performed by using Geneious 5.4 (Biomatters Ltd., Auckland, New Zealand). Sequence logos were generated by using Weblogo 3.1 (http://weblogo.threeplusone.com/). Statistical analysis was performed by using 2-tailed Fischer exact test. In the test, the numbers of FIPV-infected and FECV-infected cats were counted. For each category of FIPV or FECV infection, cats harboring viruses with or without mutations at the S1/S2 site were counted.

Furin Cleavage Assay

Fluorogenic 12-mer peptides were designed and synthesized by RS Synthesis, Louisville, KS, USA (online Technical Appendix Table 3). Purified recombinant human furin was purchased from NEB (Ipswich, MA, USA). For each reaction, 1 unit of enzyme was used in 100 μL final volume by using the reaction buffer 100 nmol/L HEPES, 0.5% Triton X-100, 1 mmol/L $CaCl_2$, 1 mmol/L 2-mercaptoethanol, pH 7.5. Peptides were diluted to 50 μmmol/L. Reactions were performed in triplicate at 30°C and fluorescence was measured with a SpectraMax fluorometer (Molecular Devices, Sunnyvale, CA, USA), enabling Vmax determination. Results for each peptide are expressed as percent cleavage by furin compared with the canonical sequence.

To perform comparative analysis of the S1/S2 cleavage site between FECVs and FIPVs, we identified cases of FIP that were confirmed postmortem by using immunohistochemistry, the standard for FIP diagnosis; archival immunohistochemistry-positive formalin-fixed tissues were used as the source of FIPV RNA. To ensure good quality sequence information from archival material, the RT-PCR amplicon size was limited to 160bp (including the S1/S2 site). This same region was then amplified from fecal material from coronavirus-positive healthy cats.

Results

FECV S1/S2

Sequencing of the S1/S2 site of 30 S sequences from FECV fecal samples revealed an extremely well-conserved motif at the amino acid level (Figure 1, panel A). In particular, arginine (R) residues are found exclusively at the most critical positions for furin recognition and cleavage (P1, P2, and P4) in all sequences analyzed (Figure 2, panel B). The P1′ position is extremely well conserved, because serine (S) is found in 100% of cases. The P5 position is also well conserved, evidenced by a clear majority of basic residues found (96.6% arginine or lysine [K]; Figure 2, panel B). At P3, limited variability is found (76.7% serine and 23.3% alanine [A]). Overall, 100% of FECV sequences analyzed contain the furin cleavage site, with a core motif of R-R-S/A-R-R-S.

FIPV S1/S2

Analysis of the S1/S2 cleavage site of FIPV sequences shows that it has much more variability, both within the narrow furin cleavage recognition motif (P4-P1) and in residues extending out of it (P8-P5 and P2′-P4′) (Figure 2A). A striking observation is that the critical positions P1 and P2 are among the most consistently mutated (Figure 3). To a lesser degree, variability extends to other positions of the cleavage motif, notably in the P1′, P3, P4, and P5 positions (Figure 2). Examination results of the entire portion of spike sequenced in this study indicate that the conserved R-R-S/A-R-R-S motif in FECV is present within a region of the spike gene that shows a high degree of variability, in contrast to other neighboring regions that are more highly conserved (online Technical Appendix Figure 1).

Correlation between FIP Status of Cats and Presence of Mutations at S1/S2

A Fisher exact test was performed to establish whether a correlation existed between the FIP status of the sampled cats and mutations at the S1/S2 site of viruses analyzed (Table 1). The test unequivocally demonstrated that there was a strong correlation ($p<0.0001$) between FIP and presence of mutations at S1/S2.

Fluorogenic Peptide Furin Cleavage Assay

To test whether the identified FIPV S1/S2 mutations have an effect on cleavability by furin, we performed an in vitro proteolytic assay. We used human furin for these experiments. Human and feline furin are very similar (96% identical) and are expected to cleave in an equivalent manner. However, feline furin has not been directly studied to any degree, and reagents are not readily available. Feline and human cells lines show identical rates of cleavage for a known furin target protein (PSCK-9), which contains an active furin

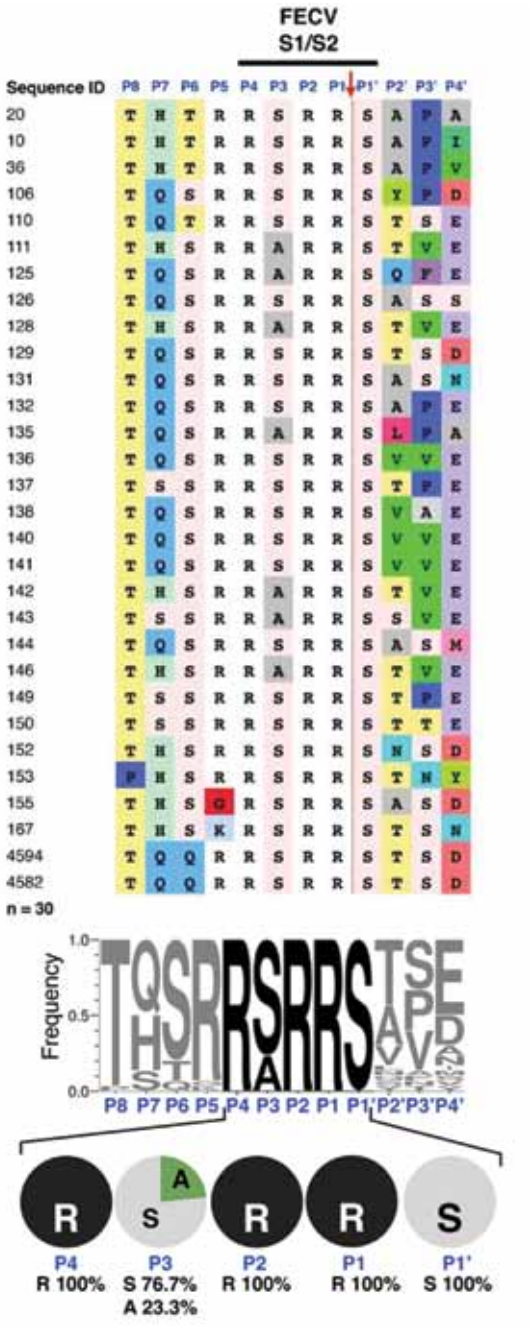

Figure 1. Sequence analysis of feline enteric coronavirus (FECV) spike S1/S2 site. RNA from 30 FECVs collected from 30 fecal samples obtained from subclinically infected cats was extracted, purified, and reverse-transcribed into cDNA. Sequencing of the spike gene was performed in a region surrounding the S1/S2 cleavage site. A) Sequence alignment. Sequence identification row (blue font): residue positions in the S1/S2 cleavage site from P8 to P4′. Red arrow indicates the site of furin cleavage. B) To visualize the diversity of residues at each position of the S1/S2 site, sequences were subjected to WebLogo 3.1 analysis (http://weblogo.threeplusone.com/create.cgi). Top: WebLogo for the 30 FECV S1/S2 sequences with the frequency of residue found at each position displayed. Bottom: summary of the diversity of residues for each position from P4 to P1′ and percentages of each amino acid represented.

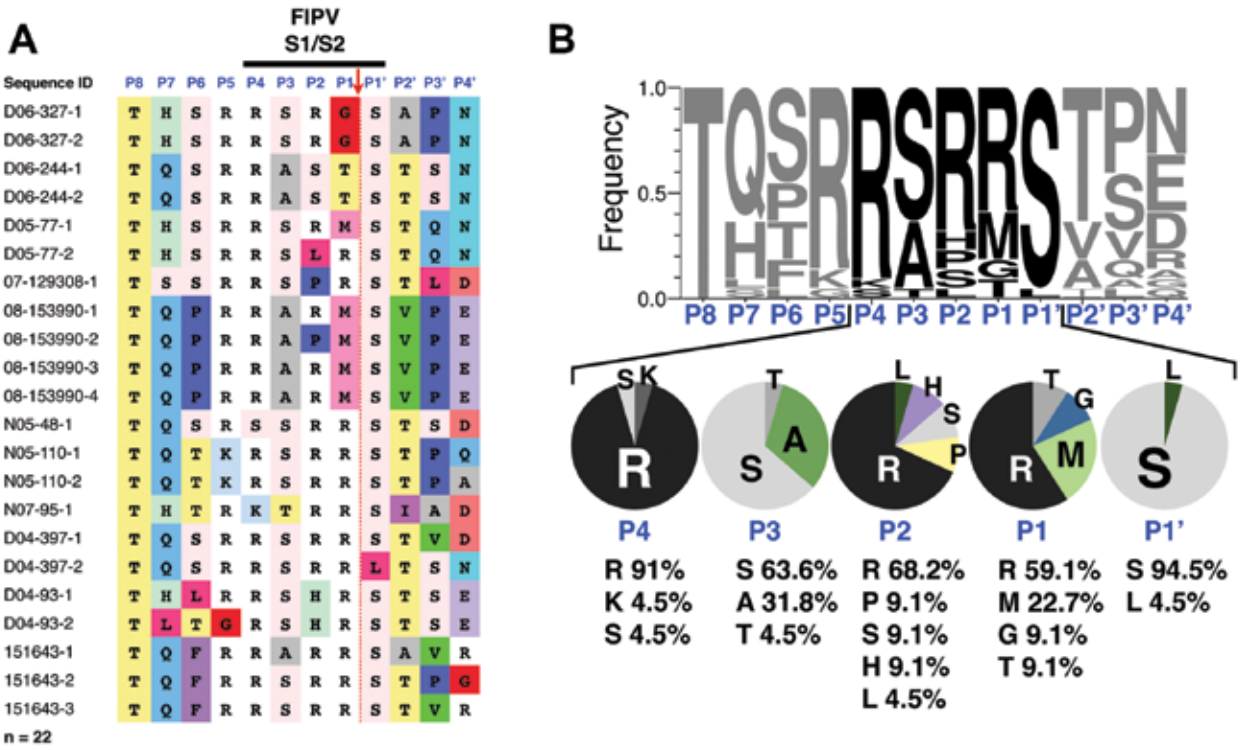

Figure 2. Sequence analysis of feline infectious peritonitis virus (FIPV) spike S1/S2 site. RNA from 22 FIPVs collected from 11 cats who had feline infectious peritonitis was extracted, purified, and reverse-transcribed into cDNA. Sequencing of the spike gene was performed in a region surrounding the S1/S2 cleavage site. A) Sequence alignment. Sequence identification row (blue font): residue positions in the S1/S2 cleavage site from P8 to P4′. Red arrow indicates the site of furin cleavage. B) To visualize the diversity of residues at each position of the S1/S2 site, sequences were subjected to WebLogo 3.1 analysis (http://weblogo.threeplusone.com/create.cgi). Top: WebLogo for the 22 FIPV S1/S2 sequences with the frequency of residue found at each position displayed. Bottom: summary of the diversity of residues for each position from P4 to P1′ and percentages of each amino acid represented.

cleavage site (online Technical Appendix Figure 2). We used fluorogenic peptides containing the canonical motif (R-R-S-R-R-S) or with substitutions from positions P1′ through P7 (Figure 4, panel A). The canonical peptide was efficiently cleaved by furin (Figure 4), with average Vmax of 235 Relative Fluorescence Units (RFU) per minute.

Within the P4-P1′ core peptide, in the canonical background, when the P1′ serine residue is changed into a leucine (L), furin cleavage is severely diminished (8% of canonical cleavage rate), a result that shows the key role of the conserved P1′ serine. Modifications of the P1 arginine in the canonical peptide, regardless of the residue tested, for example, glycine (G), methionine (M) or threonine (T), abrogate cleavage by furin (Figure 4). Modifications at the P2 arginine residue in the canonical peptide have variable effects. When P2 arginine is changed to histidine (H), there is complete inhibition (0% of canonical cleavage). When P2 is changed to leucine or serine, cleavage efficiency is reduced by ≈50% and 20%, respectively. When P2 is modified to proline (P), cleavage efficiency slightly increases to 129% of the canonical peptide (Figure 4). The P3 S-A substitution minimally enhances cleavage (Figure 4). P4 arginine is another residue position that is essential for furin cleavage. In the canonical peptide, P4 R-K substitution, there is a slight decrease in cleavage efficiency (88.7% of canonical rate). In contrast, when the P4 arginine is substituted with glycine, furin cleavage is completely abrogated (Figure 4).

For positions upstream of P4, while P5 R-K and P6 T-F modifications have moderate enhancing effects on furin cleavage (149% and 162% of canonical rate, respectively), the P7 H-Q peptide shows a substantial increase in its cleavability (186% compared with canonical). The P7 H-Q P5 R-K peptide shows that the effect of each modification can be additive (232% compared with canonical peptide) (Figure 4).

Functionally Relevant S1/S2 Mutation

To further confirm our findings, we analyzed the S1/S2 sites from viral samples taken from cats 234 and 304, who lived in the same household (Table 2). At the initial sampling in 2009 (t = 1), both cats were asymptomatic for FIP and were shedding FCoV in their feces. In samples from both cats, the S1/S2 sites had a core sequence

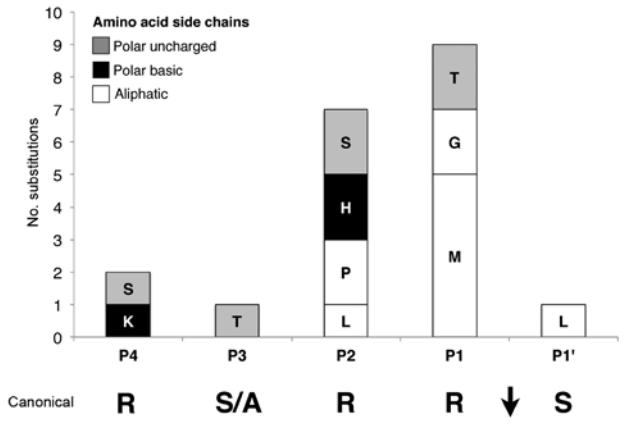

Figure 3. Amino acid substitution frequency at each position of the feline infectious peritonitis virus S1/S2 cleavage site. The histogram is based on feline infectious peritonitis virus S1/S2 WebLogo 3.1 analysis (http://weblogo.threeplusone.com/create.cgi), showing percentage of modification of residues at each position of the S1/S2 site, compared with feline enteric coronavirus S1/S2 canonical sequence consensus.

R-R-S-R-R-S consistent with the FECV consensus. Upon the second sampling in 2011/2012 (t = 2), FIP was diagnosed in cat 234. Cat 304 remained asymptomatic but continued to shed virus in feces. Notably, when the S1/S2 sequences were analyzed at the second sampling, only the cat with FIP (234) had a change in the FECV consensus sequence (a P2 R-L mutation). While exhibiting a change in the P3 residue (S-A), the virus present in cat 304 retained the conserved S1/S2 furin cleavage motif (Table 2). These data provide direct evidence of mutations in spike linked with development of FIP in cats.

Discussion

To study FIP, we have taken an alternative approach that complements earlier studies that were based on analytical outcomes of putative FIP-causing mutations and inference of their functional consequences. We focused on the S1/S2 sequence, a specific and functionally highly relevant cleavage site within the S protein, and documented mutations between asymptomatic and highly symptomatic cats that correlated strongly with FIP. We also documented a functional S1/S2 cleavage site mutation that arose in an asymptomatic cat that subsequently developed FIP.

Our sequence data show that serotype 1 FECV from feces of asymptomatic cats contain a highly conserved furin cleavage motif at the S1/S2 site, with the following narrow range of residues: $(R>>K/G)^{P5}$-$(R)^{P4}$-$(S>A)^{P3}$-$(R)^{P2}$-$(R)^{P1}$-$(S)^{P1'}$. In addition to the consensus R-X-K/R-R motif, additional flanking residues can also be consequential for furin-mediated cleavage (*26–28*). In particular, a serine (S) residue is critical in the P1′ position (*29*) and it is notable that all FECVs examined contained a P1′ S residue. The fact that the S1/S2 site is extremely well conserved is an indication that it is functionally essential for FECV replication in the enteric epithelium.

In contrast to the situation for asymptomatic cats infected with FECV, we found that sequences of FCoV sampled from tissue of confirmed FIP-positive cats consistently have mutations at the S1/S2 site. In the most critical position for furin cleavage, P1, we found that >40% of FIPVs have a mutation in the arginine residue, which is replaced by an aliphatic (methionine and glycine) or polar uncharged (threonine) residue. Overall, the distinguishing feature of FIPVs is the absence of the P1 arginine, rather that the presence of any particular residue. This is corroborated by our peptide cleavage data that demonstrate that furin cleavage is fully abrogated for all P1 substitutions tested. The next most common position mutated in FIPV is P2; >30% of the FIPVs analyzed bore mutations at this position. Most mutated residues found were aliphatic (P and L). Some sequences were substituted with a polar basic (H) or a polar uncharged (S) residue. Apart from the P2 R-P substitution, peptide cleavage data indicates that all other substitutions have an inhibiting effect on furin cleavage. Of note, for murine hepatitis virus (MHV), a betacoronavirus that also harbors an S1/S2 cleavage site in its spike protein, there is a precedent for the inhibitory effect of the introduction of a histidine in the P2 position of the cleavage site. Two well-studied strains, MHV strain A59 (MHV-A59) and the neurovirulent MHV strain JHM (MHV-JHM), have a notable difference at this site. MHV-A59 has an R-R-S-H-R-S sequence and is less efficiently cleaved than MHV-JHM, which has an R-R-A-S-S-R sequence (*18*). P4 is generally considered to be critical for furin cleavage, but we found limited variation in this residue position for the FIPVs tested and found mutation to the polar basic residue (K) or polar uncharged residue (S) in <5% of viruses. The peptide data indicates that, although introduction of a serine at P4 completely abrogates cleavage, the P4 R→K substitution has minimal effect. The FIPV P3 position showed small variation compared with FECV after the introduction of a polar uncharged residue (T) in 1 sample. For the P5 position, the only change was a slightly higher frequency of the lysine residue in samples from cats with FIPV. Peptide cleavage data indicated that the common S-A substitution found for FECV and FIPV P3 positions has only slightly increasing effect on proteolysis by furin. Furthermore, the P5 R→K substitution has an enhancing effect in the peptide cleavage assay. At P1′, the conserved polar uncharged residue (S) was retained in the majority of FIPV samples, however, the introduction of an aliphatic amino acid (L) was found. It is notable that furin cleavage has been suggested to be incompatible with a hydrophobic aliphatic side chain, with a strong preference for serine in

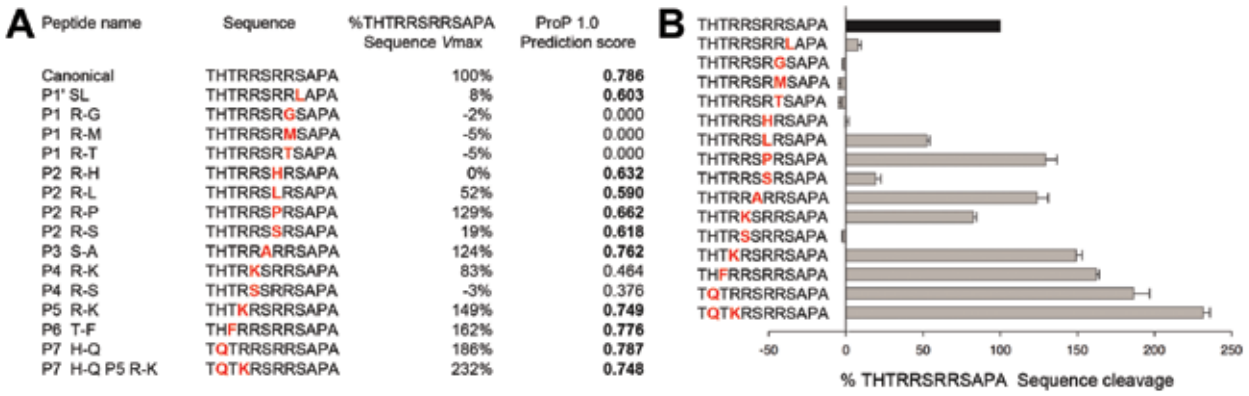

Figure 4. Furin cleavage assays of fluorogenic peptides. A) Synthetic fluorogenic peptides were generated with sequences matching consensus feline enteric coronavirus and a panel of modified sequences with substitutions (in red) found by feline infectious peritonitis virus sequencing. Peptides (50 μmol/L) were subjected to cleavage by recombinant human furin (1 U/100 μL), at pH 7.5, 30°C, and the release of fluorescence over time was measured by a spectrofluorometer enabling calculation of the Vmax of each reaction. Peptide cleavage scores generated by the ProP 1.0 server (www.cbs.dtu.dk/services/ProP/) are also displayed. B) For each modified peptide (substitutions in red), the percentage of cleavage rate compared with the canonical sequence was calculated and displayed. Cleavage assays were performed in >3 independent experiments. Error bars indicate SD for each measurement.

the P1′ position (*27*,*29*). In the D04-397-2 sample containing the P1′ L, the basic residues within the S1/S2 site remain identical to the ones found in FECV sequence; we suggest that disruption of furin cleavage is mediated by a mutation in P1′, rather than the more typical P1, P2 and/or P4 mutation. This hypothesis is supported by the peptide cleavage assay, where the P1′S-L peptide is unable to be cleaved by furin.

Overall, in terms of FIP-positive animals, we found that 10 of 11 cats harbored viruses with mutations in the furin motif R-R-S/A-R-R-S found in FECV of asymptomatic cats. For most FIP-positive cats, we sequenced viral RNA collected from different tissues (online Technical Appendix Table 1). Our data provides strong support for the internal mutation hypothesis, as the mutations are unique to individual cats.

Of note, not all tissues from the same animal carry the same mutation. In some instances, mixed populations of viruses exist within the same animal. The majority of viruses sequenced had 1 mutation, although 5 (D06-244-1, D06-244-2, 08-153990-2, N07-95-1, and D04-93-2) had 2 mutations. However, there are 2 apparent exceptions of cats harboring viruses that do not have clearly defined mutations in the furin cleavage site: samples from cat 151643 (*1*–*3*) and samples from cat N05-110 (*1*,*2*). We consider that the presence of a P6 furin cleavage in samples of cat 151643 is consistent with our hypothesis of a switch in the activating protease for the virus, because this is not typical of naturally occurring furin cleavage sites. Samples 1 and 2 from cat N05-110 harbor virus with an atypical lysine residue at P5. While unusual for FECVs, a P5 lysine residue does appear to be compatible with furin cleavage, so it remains to be determined how noteworthy a P5 lysine residue versus a P5 arginine residue is in the context of a protease switch for FIPV, or whether other mutations correlated with FIPV in the case of this cat.

As part of our study, we analyzed field samples from cats harboring FCoV at different times. In cat 234, the virus underwent a transition from FECV to FIPV, and had a functionally relevant mutation in the S1/S2 motif (P2 R-L). Cat 304, living in the same house as cat 234, remained asymptomatic. Cat 304 harbored a mutated virus, but the mutation was in a functionally irrelevant position (P3 S-A). Identification of cats with FECV in which FIP subsequently develops is challenging, and while we present a single example, we consider these data to be strong evidence that mutations at the S1/S2 site are linked to a change in the pathogenic properties of the virus, and likely to be essential for the acquisition of macrophage tropism seen in FIP.

The S1/S2 cleavage site and surrounding residues of serotype 1 FIPV S sequences were found to be systematically modified by mutations. Chang et al. recently published an extensive comparative analysis of FIP mutations at the nucleotide level by performing whole-genome sequencing of FECVs and FIPVs; the authors found a site within S (nucleotide position 23531), but outside of S1/S2, to be the most frequently mutated in FIPV (*15*). We have undertaken an analysis of the S1/S2 sites sequenced by Chang et al. and find that our hypothesis that mutation within the S1/S2 furin motif correlates with FIP in ≈64% of their samples. There are 3 differences in methodology that may explain this lack of agreement: first, we employed immunohistochemistry to confirm the diagnosis of FIP, while Chang et al. reported using postmortem examination; second, all FIP samples in this study originate from tissue,

Table 1. Status of cats sampled for feline coronavirus and mutations in spike protein cleavage site*

	FECV-infected cats	FIPV-infected cats	Total
Cats harboring viruses with ≥1 mutated S1/S2 site	2	10	12
Cats harboring viruses with an intact S1/S2 site	28	1	29
Total	30	11	41

FECV, feline enteric coronavirus; FIPV, feline infectious peritonitis; S1, receptor-binding domain of spike; S2, fusion domain of spike.

while Chang et al. included both tissue and ascites fluid; and finally, we report multiple sequences for FIP-affected cats, while Chang et al. reported a single sequence. Sequence data from samples D04-397-1 and D04-397-2 provide evidence that both FECV and FIPV populations can be identified within an affected animal. Sequence information from a single sample may not be adequate for the detection of mutated virus.

Most mutations negatively affect furin processing, but some enhance it. Given that the majority of the FIPV S proteins still harbor basic residues at the S1/S2 boundary, it could be reasoned that the mutated site becomes more open and can be cleaved by a range of other proteases. The switch in proteolytic requirements of S that we propose may offer an explanation for the crucial tropism transition during FIP. A possible consequence of the mutations is cleavability by monocytic/macrophage-specific proteases. These could be pro-protein convertases, cathepsins, or other macrophage-specific proteases. In particular, cathepsin B, matrix metalloproteases, and furin-related PCSK1 are likely to be expressed on the surface of macrophages and recognize the hallmark residues remaining or acquired in FIPV S1/S2 cleavage site (*30*). Matrix metalloprotease 9 is of particular interest because it was demonstrated to be upregulated in activated monocytes and macrophages during FIP (*31*). A shift in the entry pathway to enable virus entry at the cell surface instead of the endosome may simultaneously explain the ability of FIPV to infect macrophages and the macrophage resistance of FECV. It is also possible that the mutations in the S1/S2 region affect the heparin sulfate binding site in this region (*19*). However, heparin sulfate binding is a cell culture adaptation of the virus, and as so, its relevance to the clinical situation would appear to be unlikely.

A contrasting view to the internal mutation hypothesis to explain the genesis of FIP outbreaks is that there is a circulating FCOV other than FECV that is specific for FIP (*22*). For a complex disease process such as FIP, we and others consider it likely that there may be circulating FECVs that are closer to making the critical mutations necessary for FIP, possibly explaining paradoxical FIP outbreaks (*32*). Based on the data we present here, we conclude that mutation of the S1/S2 locus and modulation of a furin recognition site normally present in the S gene of FECVs is a critical contributing factor for development of FIP. Further studies could serve to analyze how S1/S2 mutations fit with the other mutations posited to account for FIP development.

Table 2. Sequence of FCoV spike at S1/S2 junction in cats sampled for feline coronavirus*

Time	Cat 234	Cat 304
t = 1	NHTHTRRSRR↓SAPVAV	NHTHTRRSRR↓SAPVAV
t = 2	NHTHTRRS**L**R↓SAPVAV	NHTHTRR**A**RR↓SAPVAV

*Underlines indicate nucleotide substitution. FCoV, feline coronavirus; S1/S2, cleavage site of spike protein; t = 1, both cats disease-free; ↓, cleavage site; t = 2, cat 234 feline infectious peritonitis–positive, cat 304 disease-free.

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Pneumococcal Serotypes before and after Introduction of Conjugate Vaccines, United States, 1999–2011[1]

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Serotyping data for pneumococci causing invasive and noninvasive disease in 2008–2009 and 2010–2011 from ≥43 US centers were compared with data from preconjugate vaccine (1999–2000) and postconjugate vaccine (2004–2005) periods. Prevalence of 7-valent pneumococcal conjugate vaccine serotypes decreased from 64% of invasive and 50% of noninvasive isolates in 1999–2000 to 3.8% and 4.2%, respectively, in 2010–2011. Increases in serotype 19A stopped after introduction of 13-valent pneumococcal vaccine (PCV13) in 2010. Prevalences of other predominant serotypes included in or related to PCV13 (3, 6C, 7F) also remained similar for 2008–2009 and 2010–2011. The only major serotype that increased from 2008–2009 to 2010–2011 was nonvaccine serotype 35B. These data show that introduction of the 7-valent vaccine has dramatically decreased prevalence of its serotypes and that addition of serotypes in PCV13 could provide coverage of 39% of isolates that continue to cause disease.

Infections caused by *Streptococcus pneumoniae* include meningitis, pneumonia, bacteremia, bronchitis, sinusitis, and otitis media. The World Health Organization estimated that 50%–60% of the 1.6 million deaths caused by pneumococcal infections in 2005 were in children ≤5 years of age (*1*). In the United States, ≈39,750 cases of invasive pneumococcal disease and 4,000 deaths occur annually (*2*). Since 1977, immunization with a 14-valent (now 23-valent) polysaccharide vaccine has been available in the United States for persons ≥2 years of age who have an increased risk for serious pneumococcal disease (*3*). Increasing antimicrobial drug resistance in *S. pneumoniae* during the 1990s highlighted the need for a vaccine with effectiveness in young children (*4*,*5*). In 2000, a 7-valent pneumococcal polysaccharide protein conjugate vaccine (PCV7, Prevnar; Wyeth, New York, NY, USA) for serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F became available in the United States for routine use in all children 2–23 months of age and for children 24–59 months of age who are at increased risk for pneumococcal disease (*6*).

A 30% decrease in the incidence of pneumococcal meningitis in the United States from 1998–1999 through 2004–2005 was attributed to direct vaccine effect and herd immunity, but the percentage of cases caused by non-PCV7 serotype strains, particularly 19A, increased (*7*,*8*). In 2007, the World Health Organization recommended use of PCV7 in all countries as part of routine childhood immunization (*1*), and Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA) guidance for routine use was revised to include healthy children 24–59 months of age who had not yet completed any recommended PCV7 vaccination schedule (*9*). A second pneumococcal conjugate 13-valent vaccine (PCV13) with 6 additional serotypes (1, 3, 5, 6A, 7F, and 19A) was licensed for use in the United States in March 2010 (*10*).

The large number of pneumococcal serotypes and ability of this organism to switch capsules has made prevention of disease through vaccination challenging. The recognition of new serotypes, 6C and 6D, in 2007 and 2009 brought the total number of known pneumococcal serotypes to 93 (*11*,*12*). A large portion of isolates identified as

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serotype 6A in the past were likely 6C or 6D. Monitoring of pneumococcal serotypes causing disease provides insight into pathogenesis and guidance for vaccine composition.

Although the effect of pneumococcal vaccine on invasive disease has been documented (*7*,*8*,*13*,*14*), the effect on noninvasive disease is unclear. CDC has conducted longitudinal surveillance for invasive pneumococcal disease by using 10 selected sites since the 1990s, but noninvasive disease has been excluded (*2*). Collection of diagnostic specimens that confirm the etiologic agent causing pneumonia, bronchitis, sinusitis, and otitis media can be difficult. Although isolation of *S. pneumoniae* from a normally sterile site is always considered a pathogen, recovery from a respiratory site may represent colonization. Despite these limitations, the role of surveillance that is not limited to isolates causing invasive disease is shown by recent studies reporting high case-fatality rates for noninvasive serotypes (*15*–*17*).

The purpose of this study was to examine changes in pneumococcal serotypes causing invasive and noninvasive disease in all age groups in the United State from 1999–2000 through 2010–2011. The data examined reflect longitudinal surveillance from 4 periods before and after conjugate vaccine use was implemented.

Materials and Methods

Clinical isolates of *S. pneumoniae* were collected from 45 medical centers throughout the United States during November 1, 2008–April 30, 2009, and from 43 US centers during October 1, 2010–March 31, 2011, as part of a longitudinal surveillance program. For each study period, 50 unique, consecutive pneumococcal isolates considered by the submitting laboratory to have a major clinical role were requested from each center. Isolates were not limited by patient age or specimen source. Identification of isolates was confirmed by using the bile solubility test after receipt at the central reference laboratory.

Susceptibility testing was performed on 1,750 isolates obtained during 2010–2011 and 1,946 isolates obtained during 2008–2009 by using the Clinical Laboratory and Standards Institute broth microdilution method and interpretive criteria (*18*,*19*). The results of susceptibility testing were analyzed by all 3 categories of the Clinical Laboratory and Standards Institute penicillin interpretative criteria (oral, meningitis parenteral, nonmeningitis parenteral) that have been defined since 2008. Clinical laboratories are instructed to report all 3 interpretations for penicillin results on all specimen types, with 1 exception. For cerebrospinal fluid (CSF) isolates, penicillin results are only interpreted according to the meningitis parenteral breakpoints. Therefore, CSF isolates were excluded from the analysis when nonmeningitis parenteral breakpoints were applied.

The capsular serotype of all isolates was determined by using the Quellung reaction with antisera from the Statens Serum Institut (Copenhagen, Denmark). Identity of nontypeable isolates was confirmed by using DNA probes. Serotype distributions during 2008–2009 and 2010–2011 were compared with those during 2 earlier surveillance periods representing the preconjugate vaccine (1999–2000) and the postconjugate vaccine (2004–2005) periods. This comparison required serotyping to be performed on archived penicillin-susceptible and penicillin-intermediate isolates from the 1999–2000 period (*4*). Serotyping was repeated on serogroup 6 isolates from 1999–2000 (penicillin-resistant isolates) and from 2004–2005 to detect 6C and 6D in collections for which serotyping results were published (*20*,*21*). Susceptibility data have been reported for the 1999–2000, 2004–2005, and 2010–2011 surveillance periods (*4*,*21*,*22*). The statistical significance of differences in serotype distribution for time periods, age groups, and specimen sources was assessed by using the Fisher exact test (2-tailed). This research was approved by the human subjects research review board at the University of Iowa.

Results

Patient age and specimen source distributions for the pneumococcal isolates obtained during the 4 surveillance periods were similar, with 2 exceptions (Table 1). The percentage of isolates from patients ≤5 years of age decreased from 29% in the preconjugate vaccine period to 20%–21% in the postconjugate period ($p<0.001$). The percentage of isolates from blood cultures decreased from 31% during 1999–2000 to 23%–25% during the postconjugate vaccine period ($p<0.01$).

The serotype distributions of the 2008–2009 and 2010–2011 isolates in comparison with those during 1999–2000 and 2004–2005 are shown in Table 2. The prevalence of PCV7 serotypes decreased from 55% of all isolates in 1999–2000 to 4% in 2010–2011. The percentage of serotype 19A isolates increased from 2% in 1999–2000 to 22% in 2008–2009 ($p<0.001$), and there was a slight decrease since introduction of PCV13 to 20% ($p = 0.09$). The percentages of isolates in 3 other predominant serotypes that are included in or related to PCV13 were also similar in 2008–2009 and 2010–2011: serotypes 3 (8.5% and 9.3%), 6C (7.3% and 8.5%), and 7F (5.8% and 4.9%). The only major serotype that increased from 2008–2009 through 2010–2011 was 35B (4.0% in 2008–2009 to 7.0% in 2010–2011; $p<0.001$).

In 1999–2000, PCV7 serotypes were expressed by more isolates from children ≤5 years of age (70.1%) than from other patients (48.0%; $p<0.0001$). By 2010–2011, only 1 PCV7 serotype (19F) was expressed by 7 isolates (1.9%) from children ≤5 years of age, and all PCV7 serotypes were detected among 65 isolates (4.7%) from the

Table 1. Patient demographics and penicillin susceptibility for *Streptococcus pneumoniae* isolates, United States, 1999–2011*

Characteristic	No. (%) isolates			
	Before PCV7, 1999–2000, n = 1,506	Post-PCV7, 2004–2005, n = 1,647	Post-PCV7, 2008–2009, n = 1,946	Post-PCV13, 2010–2011, n = 1,750
Medical center location				
Northeast	390 (26)	470 (29)	495 (25)	395 (23)
Midwest	394 (26)	403 (24)	395 (20)	433 (25)
Southeast	224 (15)	281 (17)	343 (18)	340 (19)
Southwest	300 (20)	265 (16)	414 (21)	396 (23)
West	198 (13)	228 (14)	299 (15)	186 (11)
Patient location				
Inpatient	846 (56)	995 (60)	1,056 (54)	987 (56)
Outpatient	654 (43)	624 (38)	817 (42)	734 (42)
Unknown	6 (0.4)	28 (2)	73 (4)	29 (2)
Age, y				
0–5†	442 (29)	328 (20)	417 (21)	367 (21)
6–20	86 (6)	137 (8)	160 (8)	173 (10)
21–64	627 (42)	769 (47)	861 (44)	824 (47)
≥65	336 (22)	350 (21)	425 (22)	376 (21)
Unknown	15 (1)	63 (4)	83 (4)	10 (0.6)
Specimen source				
Blood‡	460 (31)	406 (25)	454 (23)	428 (24)
Cerebrospinal fluid	20 (1)	14 (1)	19 (1)	16 (1)
Other sterile body fluid	–§	16 (1)	31 (2)	–§
Middle ear fluid	125 (8)	126 (8)	118 (6)	110 (6)
Lower respiratory tract	670 (44)	870 (53)	953 (49)	840 (48)
Sinus	44 (3)	91 (6)	144 (7)	147 (8)
Other	187 (12)	124 (8)	227 (12)	209 (12)
Penicillin (all isolates)				
Susceptible (MIC ≤0.06 μg/mL)¶	1,005 (67)	1,112 (68)	1,213 (62)	1,066 (61)
Intermediate (MIC 0.12–1 μg/mL)#	178 (12)	295 (18)	403 (21)	352 (20)
Resistant (MIC ≥2 μg/mL)**	323 (21)	240 (15)	330 (17)	332 (19)
Penicillin (noninvasive isolates, oral breakpoint)				
Susceptible (MIC ≤0.06 μg/mL)	652 (64)	793 (65)	828 (57)	747 (57)
Intermediate (MIC 0.12–1 μg/mL)	127 (12)	221 (18)	329 (23)	279 (21)
Resistant (MIC ≥2 μg/mL)	247 (24)	197 (16)	285 (20)	280 (21)
Penicillin (all invasive isolates, meningitis parenteral breakpoint)				
Susceptible (MIC ≤0.06 μg/mL)	353 (74)	319 (73)	385 (76)	319 (72)
Resistant (MIC ≥0.12 μg/mL)	127 (26)	117 (27)	119 (24)	125 (28)
Penicillin (invasive isolates, CSF excluded, nonmeningitis parenteral breakpoint)				
Susceptible (MIC ≤2 μg/mL)	442 (96)	400 (95)	458 (94)	392 (92)
Intermediate (MIC 4 μg/mL)	18 (4)	22 (5)	26 (5)	35 (8)
Resistant (MIC ≥8 μg/mL)	0	0	1 (0.2)	1 (0.2)

*PCV7, pneumococcal conjugate 7-valent vaccine; PCV13, pneumococcal conjugate 13-valent vaccine.
†Significant decrease from 29% in the preconjugate vaccine period to 20%–21% in the postconjugate vaccine period (p<0.001).
‡Significant decrease from 31% in 1999–2000 to 23%–25% in the postconjugate vaccine period (p<0.01).
§Other sterile body fluids were not recorded as a separate specimen source category during these 2 periods.
¶Penicillin susceptible: parenteral therapy for meningitis or oral therapy.
#Penicillin intermediate: oral therapy, resistant if parenteral therapy for meningitis.
**Penicillin resistant: parenteral therapy for meningitis or oral therapy.

other age groups. The percentage of serotype 19A isolates among patients ≤5 years of age peaked in 2008–2009 at 36.7%, and the percentage was 34.1% in 2010–2011. The prevalence of serotype 19A (34.1% vs. 16.3%; p<0.0001) and 35B (10.6% vs. 6.1%; p = 0.004) isolates was higher and the percentage of serotype 3 isolates (4.1% vs. 10.7%; p<0.0001) was lower for children ≤5 years of age than for other age groups in 2010–2011.

Among 444 isolates from invasive specimens (blood and CSF) in 2010–2011 (Table 3), the predominant serotypes were 19A (18.2%), 7F (12.4%), 3 (9.5%), 6C (7.9%), 22F (6.3%), and 23A (5.2%). Serotype 7F isolates were more commonly recovered from invasive than noninvasive specimens (12.4% vs. 2.4%; p<0.001); serotype 35B isolates were recovered more often from noninvasive than invasive specimens (8.0% vs. 4.3%; p = 0.007). Rates of recovery from invasive and noninvasive specimens were similar for other serotypes. The distribution of major serotypes in isolates recovered during 2010–2011 from lower respiratory tract specimens (19A [18.3%], 3 [9.5%], 6C [9.4%], and 35B [8.0%]) was similar to all specimen sources combined. Serotype 19A isolates were more prevalent among middle ear fluid specimens than among other specimen types (36.4% vs. 18.9%; p<0.001).

Penicillin-intermediate and penicillin-resistant *S. pneumoniae* isolates defined by the nonmeningitis parenteral

Table 2. Serotype distribution of *Streptococcus pneumoniae* isolates, United States, 1999–2011*

	No. (%) isolates							
	All age groups				Children ≤5 y of age			
Serotype	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011
PCV7								
4†	69 (4.6)	34 (2.1)	7 (0.4)	6 (0.3)	14 (3.1)	0	0	0
6B†	157 (10.4)	32 (1.9)	8 (0.4)	11 (0.6)	73 (16.3)	7 (2.1)	0	0
9V†	77 (5.1)	21 (1.3)	5 (0.3)	1 (0.1)	19 (4.2)	3 (0.9)	0	0
14†	151 (10.0)	13 (0.8)	7 (0.4)	1 (0.1)	67 (15.0)	0	1 (0.2)	0
18C†	51 (3.4)	10 (0.6)	2 (0.1)	3 (0.2)	23 (5.2)	0	0	0
19F†	181 (12.0)	116 (7.0)	61 (3.1)	47 (2.7)	69 (15.4)	26 (7.9)	15 (3.6)	7 (1.9)
23F†	135 (9.0)	40 (2.4)	5 (0.3)	3 (0.2)	45 (10.1)	8 (2.4)	2 (0.5)	0
Total	821 (54.5)	266 (16.1)	95 (4.9)	72 (4.1)	310 (70.1)	44 (13.4)	18 (4.3)	7 (1.9)
Additional serotypes in PCV13								
1†	17 (1.1)	12 (0.7)	10 (0.5)	2 (0.1)	3 (0.7)	2 (0.6)	1 (0.2)	0
3†	127 (8.4)	184 (11.2)	165 (8.5)	163 (9.3)	14 (3.1)	23 (7.0)	17 (4.1)	15 (4.1)
5†	0	2 (0.1)	1 (0.1)	1 (0.1)	0	0	1 (0.2)	0
6A	87 (5.8)	78 (4.7)	26 (1.3)	7 (0.4)	35 (7.8)	19 (5.8)	5 (1.2)	1 (0.3)
7F†	21 (1.4)	29 (1.8)	113 (5.8)	86 (4.9)	2 (0.5)	2 (0.6)	14 (3.4)	4 (1.1)
19A†	33 (2.2)	239 (14.5)	434 (22.3)	350 (20.0)	12 (2.7)	83 (25.3)	153 (36.7)	125 (34.1)
Total	285 (18.9)	544 (33.0)	749 (38.5)	609 (34.8)	66 (14.9)	129 (39.3)	191 (45.8)	145 (39.5)
PCV related								
6C	12 (0.8)	37 (2.3)	141 (7.3)	148 (8.5)	2 (0.5)	2 (0.6)	29 (7.0)	32 (8.7)
9N	19 (1.3)	14 (0.9)	31 (1.6)	34 (1.9)	4 (0.9)	1 (0.3)	2 (0.5)	2 (0.5)
23A	10 (0.7)	47 (2.8)	86 (4.4)	81 (4.6)	0	9 (2.7)	18 (4.3)	10 (2.7)
23B	2 (0.1)	25 (1.5)	58 (3.0)	44 (2.5)	0	4 (1.2)	14 (3.3)	11 (3.0)
Other	18 (1.2)‡	36 (2.2)§	22 (1.1)¶	31 (1.8)#	5 (1.1)‡	4 (1.2)§	5 (1.2)¶	1 (0.3)#
Total	61 (4.1)	159 (9.7)	338 (17.4)	338 (19.3)	11 (2.5)	20 (6.1)	68 (16.3)	56 (15.3)
Non-PCV								
11A†	52 (3.5)	101 (6.1)	77 (4.0)	82 (4.7)	3 (0.7)	16 (4.9)	12 (2.9)	14 (3.8)
12F	17 (1.1)	29 (1.8)	8 (0.4)	14 (0.8)	1 (0.2)	2 (0.6)	1 (0.2)	0
15A	7 (0.5)	45 (2.7)	78 (4.0)	53 (3.0)	3 (0.7)	10 (3.0)	16 (3.8)	3 (0.8)
15B†	7 (0.5)	38 (2.3)	39 (2.0)	58 (3.3)	4 (0.9)	12 (3.7)	14 (3.4)	22 (6.0)
15C	12 (0.8)	30 (1.8)	29 (1.5)	43 (2.5)	3 (0.7)	9 (2.7)	13 (3.1)	17 (4.6)
16F	17 (1.1)	38 (2.3)	57 (2.9)	39 (2.2)	0	5 (1.5)	5 (1.2)	5 (1.4)
22F†	36 (2.4)	77 (4.7)	95 (4.9)	84 (4.8)	3 (0.7)	14 (4.3)	10 (2.4)	20 (5.4)
35B	30 (2.0)	73 (4.4)	78 (4.0)	123 (7.0)	6 (1.3)	24 (7.3)	20 (4.8)	39 (10.6)
Other	113 (7.5)**	217 (13.2)††	266 (13.7)‡‡	190 (10.9)§§	19 (4.3)**	41 (12.5)††	45 (10.8)‡‡	33 (9.0)§§
NT	48 (3.2)	30 (1.8)	37 (1.9)	45 (2.6)	13 (2.9)	2 (0.6)	4 (1.0)	6 (1.6)
Total	339 (22.5)	678 (41.2)	764 (39.3)	731 (41.8)	55 (12.4)	135 (41.1)	140 (33.6)	159 (43.3)
All	1,506	1,647	1,946	1,750	442	328	417	367

*PCV7, pneumococcal conjugate 7-valent vaccine; PCV13, pneumococcal conjugate 13-valent vaccine; NT, nontypeable; ch, children.
†Serotypes in 23-valent polysaccharide pneumococcal vaccine.
‡Other low prevalence serotypes (no. isolates): in 1999–2000, serotype 7C (2), 9A (8, 2 ch), 9L (2), 18F (2), 18B (4, 3 ch).
§In 2004–2005, serotype 6D (1), 7A (1), 7C (8), 9A (5, 1 ch), 9L (5), 18F (4), 18A (3, 1 ch), 18B (5, 1 ch), 19B (3, 1 ch), 19C (1).
¶In 2008–2009, serotype 7C (12, 5 ch), 9A (3), 9L (5), 19B (1), 19C (1).
#In 2010–2011, serotype 6D (1), 7B (1 ch), 7C (21), 9A (2), 9L (5), 19B (1).
**In 1999–2000, serotype 8 (7), 10A (14, 3 ch), 11B (1), 13 (8, 1 ch), 16A (1, 1 ch), 17F (2, 1 ch), 20 (3), 21 (4, 3 ch), 25A (9, 3 ch), 25F (1), 28F (1), 28A (4), 29 (3, 1 ch), 31 (13), 33F (5, 2 ch), 33A (7, 1 ch), 34 (4), 35F (18, 2 ch), 35C (1), 36 (1), 37 (1), 38 (2), 45 (2, 1 ch), 47 (1).
††In 2004–05, serotype 2 (1), 8 (7), 10F (1), 10A (23, 1 ch), 11D (1), 12B (2), 13 (5), 15F (2), 16A (2, 1 ch), 17F (11), 20 (7, 2 ch), 21 (6, 1 ch), 24F (1), 25A (3, 1 ch), 28F (2), 28A (2), 29 (8, 6 ch), 31 (36, 6 ch), 33F (16, 4 ch), 33A (6, 3 ch), 34 (11, 1 ch), 35F (34, 6 ch), 35A (15, 4 ch), 38 (12, 3 ch), 39 (1 ch), 40 (1), 48 (1 ch).
‡‡In 2008–2009, serotype 8 (13, 1 ch), 10F (1), 10A (28, 4 ch), 10B (1), 11D (1), 13 (3), 16A (1), 17F (28, 6 ch), 20 (14, 1 ch), 21 (20, 9 ch), 22A (1 ch), 25A (6, 1 ch), 28A (2), 29 (7, 2 ch), 31 (35, 2 ch), 32F (1), 33F (23, 2 ch), 33A (17, 3 ch), 33B (3, 1 ch), 34 (23, 4 ch), 35F (25, 6 ch), 35A (2), 35C (2, 1 ch), 37 (3), 38 (3), 42 (3, 1 ch).
§§In 2010–2011, serotype 8 (10), 10A (26, 5 ch), 13 (2), 17F (15, 3 ch), 20 (6), 21 (15, 9 ch), 24A (2, 1 ch), 28A (1), 29 (3), 31 (28, 2 ch), 33F (16, 3 ch), 33A (18, 3 ch), 34 (15, 2 ch), 35F (17, 4 ch), 38 (15), 45 (1 ch).

breakpoint (MIC ≥4 μg/mL) were rare for all study periods (4%–8% of invasive non-CSF isolates) (Table 1). All of these invasive isolates in 2010–2011 (n = 36) and 93% (25 of 27) in 2008–2009 were serotype 19A.

The percentage of penicillin-nonsusceptible *S. pneumoniae* (PNSP) (MIC ≥0.12 μg/mL, resistant for meningitis; intermediate and resistant categories for oral therapy of non-meningitis infections) increased from 33% in 1999–2000 to 39% in 2010–2011 (p<0.001). Predominant PNSP serotypes in 2010–2011 were 19A (41%), 35B (15%), 6C (11%), 23A (8%), 15A (6%), and 19F (4%). The change in distribution of predominant PNSP serotypes over time is shown in the Figure. Serotype 19A isolates increased from 5% of PNSP in 1999–2000 to 29% in 2004–2005 and 46% in 2008–2009, and there was a slight decrease to 41% of PNSP in 2010–2011. (PCV13, which includes this serotype, was introduced

Table 3. Serotype distribution of *Streptococcus pneumoniae* noninvasive and invasive isolates, United States, 1999–2011*

	No. (%) isolates							
	Noninvasive				Invasive			
Serotype	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011
PCV7								
4†	20 (1.9)	11 (0.9)	5 (0.4)	2 (0.2)	49 (10.2)	23 (5.3)	2 (0.4)	4 (0.9)
6B†	104 (10.1)	25 (2.1)	5 (0.4)	7 (0.5)	53 (11.0)	7 (1.6)	3 (0.6)	4 (0.9)
9V†	47 (4.6)	12 (1.0)	5 (0.4)	0	30 (6.3)	9 (2.1)	0	1 (0.2)
14†	69 (6.7)	9 (0.7)	5 (0.4)	0	82 (17.1)	4 (0.9)	2 (0.4)	1 (0.2)
18C†	25 (2.4)	6 (0.5)	0	2 (0.2)	26 (5.4)	4 (0.9)	2 (0.4)	1 (0.2)
19F†	144 (14.0)	95 (7.8)	55 (3.8)	41 (3.1)	37 (7.7)	21 (4.8)	6 (1.2)	6 (1.4)
23F†	105 (10.2)	33 (2.7)	4 (0.3)	3 (0.2)	30 (6.3)	7 (1.6)	1 (0.2)	0
Total	514 (50.1)	191 (15.8)	79 (5.5)	55 (4.2)	307 (64.0)	75 (17.2)	16 (3.2)	17 (3.8)
Additional serotypes in PCV13								
1†	9 (0.9)	6 (0.5)	4 (0.3)	0	8 (1.7)	6 (1.4)	6 (1.2)	2 (0.5)
3†	108 (10.5)	145 (12.0)	126 (8.7)	121 (9.3)	19 (4.0)	39 (8.9)	39 (7.7)	42 (9.5)
5†	0	2 (0.2)	0	1 (0.1)	0	0	1 (0.2)	0
6A	56 (5.5)	53 (4.4)	21 (1.5)	4 (0.3)	31 (6.5)	25 (5.7)	5 (1.0)	3 (0.7)
7F†	10 (1.0)	11 (0.9)	38 (2.6)	31 (2.4)	11 (2.3)	18 (4.1)	75 (14.9)	55 (12.4)
19A†	24 (2.3)	163 (13.5)	330 (22.9)	269 (20.6)	9 (1.9)	76 (17.4)	104 (20.6)	81 (18.2)
Total	207 (20.2)	380 (31.4)	519 (36.0)	426 (32.6)	78 (16.3)	164 (37.6)	230 (45.6)	183 (41.2)
PCV related								
6C	10 (1.0)	30 (2.5)	104 (7.2)	113 (8.7)	2 (0.4)	7 (1.6)	37 (7.3)	35 (7.9)
9N	14 (1.4)	12 (1.0)	22 (1.5)	20 (1.5)	5 (1.3)	2 (0.5)	9 (1.8)	14 (3.2)
23A	10 (1.0)	37 (3.1)	65 (4.5)	58 (4.4)	0	10 (2.3)	21 (4.2)	23 (5.2)
23B	2 (0.2)	22 (1.8)	50 (3.5)	36 (2.8)	0	3 (0.7)	8 (1.6)	8 (1.8)
Other	12 (1.2)‡	26 (2.1)§	13 (0.9)¶	23 (1.8)#	6 (2.3)‡	10 (2.3)§	9 (1.8)¶	8 (1.8)#
Total	48 (4.7)	127 (10.5)	254 (17.6)	250 (19.1)	13 (2.7)	32 (7.3)	84 (16.7)	88 (19.8)
Non-PCV								
11A†	39 (3.8)	89 (7.4)	64 (4.4)	71 (5.4)	13 (2.7)	12 (2.8)	13 (2.6)	11 (2.5)
12F	4 (0.4)	4 (0.3)	–	3 (0.2)	13 (2.7)	25 (5.7)	8 (1.6)	11 (2.5)
15A	6 (0.6)	35 (2.9)	67 (4.7)	36 (2.8)	1 (0.2)	10 (2.3)	11 (2.2)	17 (3.8)
15B†	5 (0.5)	27 (2.2)	35 (2.4)	52 (4.0)	2 (0.4)	11 (2.5)	4 (0.8)	6 (1.4)
15C	9 (0.9)	21 (1.7)	24 (1.7)	36 (2.8)	3 (0.6)	9 (2.1)	5 (1.0)	7 (1.6)
16F	11 (1.1)	27 (2.2)	44 (3.1)	31 (2.4)	6 (1.3)	11 (2.5)	13 (2.6)	8 (1.8)
22F†	19 (1.9)	50 (4.1)	53 (3.7)	56 (4.3)	17 (3.5)	27 (6.2)	42 (8.3)	28 (6.3)
35B	28 (2.7)	68 (5.6)	70 (4.9)	104 (8.0)	2 (0.4)	5 (1.2)	8 (1.6)	19 (4.3)
Other	94 (9.2)**	165 (13.6)††	198 (13.7)‡‡	144 (11.0)§§	19 (4.0)**	52 (11.9)††	68 (13.5)‡‡	46 (10.4)§§
NT	42 (4.1)	27 (2.2)	53 (2.4)	42 (3.2)	6 (1.3)	3 (0.7)	2 (0.4)	3 (0.7)
Total	257 (25.0)	513 (42.4)	590 (40.9)	575 (44.0)	82 (17.1)	165 (37.8)	174 (34.5)	156 (35.1)
All	1,026	1,211	1,442	1,306	480	436	504	444

*PCV7, pneumococcal conjugate 7-valent vaccine; PCV13, pneumococcal conjugate 13-valent vaccine; NT, nontypeable; non, noninvasive; inv, invasive.
†Serotypes in 23-valent polysaccharide pneumococcal vaccine.
‡Other low prevalence serotypes (no. isolates): in 1999–2000, serotype 7C (2 non), 9A (4 non; 4 inv), 9L (2 non), 18F (1 non; 1 inv), 18B (3 non; 1 inv).
§In 2004–2005, serotype 6D (1 non), 7A (1 non), 7C (5 non; 3 inv), 9A (4 non; 1 inv), 9L (4 non; 1 inv), 18F (2 non; 2 inv), 18A (3 non), 18B (5 non), 19B (3 inv), 19C (1 non).
¶In 2008–2009, serotype 7C (9 non; 3 inv), 9A (2 non; 1 inv), 9L (1 non; 4 inv), 19B (1 inv), 19C (1 non).
#In 2010–2011, serotype 6D (1 non), 7B (1 non), 7C (15 non; 6 inv), 9A (1 non; 1 inv), 9L (4 non; 1 inv), 19B (1 non).
**In 1999–2000, serotype 8 (3 non; 4 inv); 10A (13 non, 1 inv); 11B (1 non); 13 (8 non); 16A (1 non); 17F (2 non); 20 (2 non; 1 inv); 21 (3 non; 1 inv); 25A (7 non; 2 inv); 25F (1 non); 28F (1 non); 28A (3 non; 1 inv); 29 (1 non; 2 inv); 31 (12 non; 1 inv); 33F (4 non; 1 inv); 33A (6 non; 1 inv); 34 (4 non); 35F (15 non; 3 inv); 35C (1 inv); 36 (1 non); 37 (1 non); 38 (2 non); 45 (2 non); 47 (1 non).
††In 2004–2005, serotype 2 (1 non), 8 (3 non; 4 inv), 10F (1 inv), 10A (17 non; 6 inv), 11D (1 non), 12B (1 non; 1 inv), 13 (3 non; 2 inv), 15F (1 non; 1 inv), 16A (2 non), 17F (7 non; 4 inv), 20 (4 non; 3 inv), 21 (6 non), 24F (1 non), 25A (2 non; 1 inv), 28F (2 inv), 28A (1 non; 1 inv), 29 (8 non), 31 (32 non; 4 inv), 33F (10 non; 6 inv), 33A (2 non; 4 inv), 34 (9 non; 2 inv), 35F (28 non; 6 inv), 35A (15 non), 38 (8 non; 4 inv), 39 (1 non), 40 (1 non), 48 (1 non).
‡‡In 2008–2009, serotype 8 (6 non; 7 inv), 10F (1 inv), 10A (23 non; 5 inv), 10B (1 inv), 11D (1 non), 13 (2 non; 1 inv), 16A (1 inv), 17F (24 non; 4 inv), 20 (7 non; 7 inv), 21 (19 non; 1 inv), 22A (1 non), 25A (5 non; 1 inv), 28A (2 non), 29 (6 non; 1 inv), 31 (27 non; 8 inv), 32F (1 inv), 33F (11 non; 12 inv), 33A (11 non; 6 inv), 33B (3 non), 34 (18 non; 5 inv), 35F (21 non; 4 inv), 35A (2 non), 35C (2 non), 37 (3 non), 38 (1 non; 2 inv), 42 (3 non).
§§In 2010–2011, serotype 8 (4 non; 6 inv), 10A (21 non; 5 inv), 13 (2 non), 17F (13 non; 2 inv), 20 (3 non; 3 inv), 21 (14 non; 1 inv), 24A (2 non), 28A (1 non), 29 (3 non), 31 (24 non; 4 inv), 33F (10 non; 6 inv), 33A (9 non; 9 inv), 34 (10 non; 5 inv), 35F (15 non; 2 inv), 38 (12 non; 3 inv), 45 (1 non).

in March 2010; see Discussion.) Serotype 35B isolates also increased from 4% of PNSP in 1999–2000 to 15% of PNSP in 2010–2011.

The rate of penicillin-resistant *S. pneumoniae* (PRSP) (MIC ≥2 μg/mL, oral therapy) among all isolates decreased from 21% in 1999–2000 (Table 1) to 15% in 2004–2005 (p<0.0001), followed by increases to reach 19% in 2010–2011 (p = 0.0007). Most PRSP isolates during 2010–11 were serotype 19A (75.3%), 35B (11.1%), or 19F (7.2%). Serotype 19A isolates increased from 2% of PRSP in 1999–2000 to 35% in 2004–2005 and 80% in 2008–2009, and there was a slight decrease to 75% of PRSP in 2010–2011. Serotype 35B isolates have also increased from 1% of PRSP in 1999–2000 to 11% of PRSP in 2010–2011.

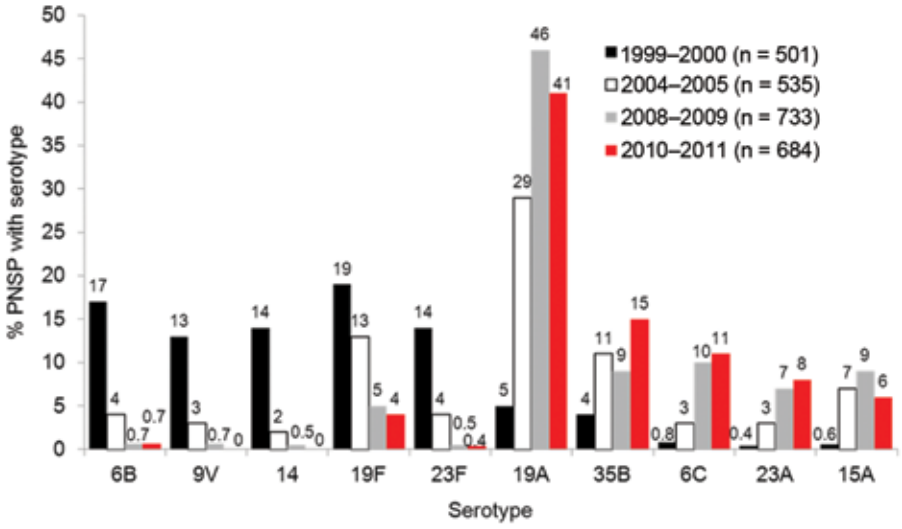

Figure. Serotype distribution of penicillin-nonsusceptible *Streptococcus pneumoniae* (PNSP) (MIC ≥0.12 μg/mL) United States, 1999–2011. Values above bars are percentages.

The rate of penicillin-intermediate *S. pneumoniae* (PISP) (MIC 0.12–1 μg/mL, oral therapy) among all isolates increased from 12% in 1999–2000 to 20% in 2010–2011 ($p<0.0001$). The most common serotypes of these penicillin-intermediate strains in 2010–2011 were 6C (21%), 35B (19%), 23A (16%), 15A (11%), and 19A (9%).

For each time period, a larger percentage of invasive isolates (72%–76%) were penicillin susceptible (MIC ≤0.06 μg/mL, meningitis parenteral breakpoint) in comparison to those from noninvasive sites (57%–65%) (Table 1). Separate serotype data for noninvasive and invasive PNSP isolates are shown in Table 4. In 2010–2011, percentages of PCV7 and non-PCV serotypes were higher among noninvasive than invasive PNSP isolates (6.1% vs. 1.6%; $p = 0.04$ and 32% vs. 21%; $p = 0.01$), and the prevalence of isolates with additional serotypes in PCV13 was similar (42% and 46%). PCV7-related serotypes comprised a larger percentage of the PNSP invasive isolates (32% vs. 20%; $p = 0.004$). The prevalence of individual serotypes among 2010–2011 PNSP isolates was similar for noninvasive and invasive isolates, except for 3 serotypes. The percentage of serotypes 23A and 23B among PNSP invasive isolates in 2010–2011 was higher than in noninvasive isolates (15% vs. 6.6%; $p = 0.003$, and 4.8% vs. 1.3%; $p = 0.02$). The percentage of serotype 19F among PNSP noninvasive isolates in 2010–2011 was higher than in invasive isolates (4.8% vs. 0.8%; $p = 0.04$). Serotype distribution over time for noninvasive and invasive isolates obtained from children ≤5 years of age are shown in Table 5.

Discussion

This longitudinal study demonstrates the effectiveness of PCV7 in children ≤5 years of age by a decrease in PCV7 serotypes from 70% of isolates in during 1999–2000 to only 1.9% during 2010–2011. Among other age groups, for which routine PCV7 use is not recommended, an indirect vaccine effect is apparent; the percentage of PCV7 serotypes decreased from 48% to 4.9%. Although PCV13 (PCV7 plus serotypes 1, 3, 5, 6A, 7F, and 19A) has been licensed for administration to children and adults ≥50 years of age, the Advisory Committee on Immunization Practices has not yet issued guidance for use in adults (*23*). Fewer cases and lower cost have been projected as potential benefits if PCV13 were given to the older US population (*24*).

As PCV7 serotypes decreased, serotype 19A strains began causing a higher percentage of invasive and noninvasive disease (*21*,*25*). Serotype 19A was the predominant serotype in our study during the post-PCV periods and accounted for 20% of isolates and 41% of PNSP during 2010–2011. The prevalence of serotype 19A strains was similar for invasive and noninvasive disease. Population-based CDC surveillance of invasive pneumococcal disease in the United States during 2007 reported that 40% of PNSP were serotype 19A (*13*). Recovery of serotype 19A isolates during 2010–2011 from middle ear fluid was much higher in our study than for other specimen sources (36.4% vs. 18.9%). A lower nasopharyngeal carriage rate of serotype 19A in PCV13-vaccinated children in France with acute otitis media suggests a decrease in serotype 19A disease will follow (*26*). Our study showed a slight decrease in the relative number of serotype 19A clinical isolates since introduction of PCV13 that should be apparent as a major trend by the 2012–2013 respiratory season.

The prevalence of serotype 3 strains in this study was fairly constant among invasive and noninvasive specimen types, and there was no evidence of change apparent since PCV13 introduction. High mortality rates have been associated with invasive disease caused by serotype 3 (*27*,*28*). The lower occurrence of serotype 3 isolates among children observed in the present study has also been reported in Germany (*29*).

Table 4. Serotype distribution of pencillin-nonsusceptible noninvasive and invasive pneumococcal isolates, United States, 1999–2011*

	No. (%) isolates							
	Noninvasive				Invasive			
	Pencillin intermediate and resistant (oral breakpoint), MIC ≥0.12 µg/mL				Pencillin resistant (meningitis breakpoint), MIC ≥0.12 µg/mL			
Serotype	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011
PCV7								
4†	1 (0.3)	2 (0.5)	0	0	1 (0.8)	0	0	0
6B†	63 (16.8)	15 (3.6)	3 (0.5)	4 (0.7)	24 (18.9)	5 (4.3)	2 (1.7)	1 (0.8)
9V†	44 (11.8)	11 (2.6)	5 (0.8)	0	23 (18.1)	7 (6.0)	0	0
14†	38 (10.2)	6 (1.4)	3 (0.5)	0	33 (26.0)	3 (2.6)	1 (0.8)	0
18C†	2 (0.5)	–	0	0	1 (0.8)	0	0	0
19F†	85 (22.7)	56 (13.4)	38 (6.2)	27 (4.8)	10 (7.9)	12 (10.3)	2 (1.7)	1 (0.8)
23F†	58 (15.5)	16 (3.8)	3 (0.5)	3 (0.5)	13 (10.2)	3 (2.6)	1 (0.8)	0
Total	291 (77.8)	106 (25.4)	52 (8.5)	34 (6.1)	105 (82.7)	30 (25.6)	6 (5.0)	2 (1.6)
Additional serotypes in PCV13								
1†	0	1 (0.2)	0	0	0	0	0	0
3†	2 (0.5)	5 (1.2)	0	2 (0.4)	0	1 (0.9)	0	0
6A	17 (4.5)	32 (7.7)	17 (2.3)	3 (0.5)	6 (4.7)	17 (14.5)	2 (1.7)	1 (0.8)
7F†	0	6 (1.4)	0	1 (0.2)	0	0	1 (0.8)	1 (0.8)
19A†	16 (4.3)	111 (26.6)	275 (44.8)	227 (40.6)	7 (5.5)	44 (37.6)	59 (49.6)	55 (44.0)
Total	35 (9.4)	149 (35.6)	292 (47.6)	233 (41.7)	13 (10.2)	62 (53.0)	62 (52.1)	57 (45.6)
PCV-related								
6C	3 (0.8)	12 (2.9)	61 (9.9)	63 (11.3)	1 (0.8)	2 (1.7)	13 (10.9)	14 (11.2)
9A	2 (0.5)	4 (1.0)	2 (0.3)	1 (0.2)	4 (3.2)	0	1 (0.8)	1 (0.8)
19B	0	0	0	1 (0.2)	0	1 (0.9)	1 (0.8)	0
23A	2 (0.5)	13 (3.1)	39 (6.4)	37 (6.6)	0	4 (3.4)	13 (10.9)	19 (15.2)
23B	0	5 (1.2)	12 (2.0)	7 (1.3)	0	0	1 (0.8)	6 (4.8)
Other	2 (0.5)‡	2 (0.5)§	1 (0.2)¶	2 (0.4)#	0	0	0	0
Total	9 (2.4)	36 (8.6)	115 (18.7)	111 (19.9)	5 (3.9)	7 (6.0)	29 (24.4)	40 (32.0)
Non-PCV								
11A†	0	5 (1.2)	2 (0.3)	7 (1.3)	0	1 (0.9)	0	0
15A	3 (0.8)	27 (6.5)	56 (9.1)	29 (5.1)	0	8 (6.8)	9 (7.6)	11 (8.8)
15B†	0	1 (0.2)	6 (1.0)	22 (3.9)	0	0	1 (0.8)	0
15C	0	3 (0.7)	7 (1.1)	12 (2.1)	0	0	3 (2.5)	1 (0.8)
35B	19 (5.1)	56 (13.4)	56 (9.1)	89 (15.9)	1 (0.8)	5 (4.3)	7 (5.9)	13 (10.4)
Other	4 (1.1)**	26(6.2)††	19 (3.1)‡‡	11 (2.0)§§	1 (0.8)**	2 (1.7)††	2 (1.7)‡‡	0
NT	13 (3.5)	9 (2.2)	9 (1.4)	11 (2.0)	2 (1.6)	2 (1.7)	0	1 (0.8)
Total	39 (10.4)	127 (30.4)	155 (25.2)	181 (32.4)	4 (3.1)	18 (15.4)	22 (18.5)	26 (20.8)
All serotypes	374	418	614	559	127	117	119	125

*PCV7, pneumococcal conjugate 7-valent vaccine; PCV13, pneumococcal conjugate 13-valent vaccine; NT, nontypeable; non, noninvasive; inv, invasive.
†Serotypes in 23-valent polysaccharide pneumococcal vaccine.
‡Other low prevalence serotypes (no. isolates): in 1999–2000, serotype 9N (1 non), 18 F (1 non).
§In 2004–2005, serotype 18B (1 non), 19C (1 non).
¶In 2008–2009, serotype 9N (1 non).
#In 2010–2011, serotype 7C (1 non), 9N (1 non).
**In 1999–00, serotype 16F (2 non), 22F (1 non), 29 (1 non, 1 inv).
††In 2004–2005, serotype 15F (1 non), 16F (1 non), 20 (1 non), 21 (1 non), 22F (1 inv), 29 (8 non), 31 (1 non), 33F (1 inv), 35A (9 non), 35F (2 non), 39 (1 non), 48 (1 non).
‡‡In 2008–09, serotype 8 (1 non), 11D (1 non), 17F (2 non), 21 (1 non), 22F (1 inv), 22A (1 non), 25A (1 non), 29 (6 non, 1 inv), 33F (1 non), 34 (1 non), 35A (1 non), 42 (3 non).
§§In 2010–11, serotype 16F (2 non), 21 (2 non), 29 (1 non), 31 (2 non), 33F (1 non), 34 (1 non), 38 (2 non).

An increase in 7F strains from 1.4% during 1999–2000 to 5.8% during 2008–2009 ($p<0.001$) did not change in the post-PCV13 period. A lower rate of nasopharyngeal colonization with 7F strains in children in France vaccinated with PCV13 is predictive of a future decrease in 7F disease (*26*). Serotype 7F was the only serotype recovered in more invasive than noninvasive specimen types in the current study (12.4% vs. 4.9% during 2010–2011). A study in Germany of invasive pneumococcal disease in children <16 years of age demonstrated the highest risk for severe and fatal outcomes for infection with serotype 7F (*27*). However, a meta-analysis associating serotypes with deaths from bacteremic pneumonia, including a small number of cases in children, reported a decreased risk for death for serotype 7F (*28*).

Serotypes 1 and 5 were included in PCV13 because these strains are major causes of invasive disease in children outside the United States (*30*). In our study, only a small number of these serotypes were obtained. During 2010–2011, the 2 serotype 1 isolates were from blood cultures, and the only serotype 5 isolate was from a lower respiratory tract specimen.

Table 5. Serotype distribution of pneumococcal noninvasive and invasive isolates from children ≤5 y of age, United States, 1999–2011*

	Noninvasive					Invasive				
Serotype	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011	Pen NS, 2010–2011	Before PCV7, 1999–2000	Post-PCV7, 2004–2005	Post-PCV7, 2008–2009	Post-PCV13, 2010–2011	Pen NS, 2010–2011
PCV7										
4†	4 (1.3)	0	0	0	0	10 (7.0)	0	0	0	0
6B†	46 (15.1)	5 (1.8)	0	0	0	27 (19.0)	2 (3.8)	0	0	0
9V†	14 (4.6)	1 (0.4)	0	0	0	5 (3.5)	2 (3.8)	0	0	0
14†	28 (9.2)	0	1 (0.3)	0	0	39 (27.5)	0	0	0	0
18C†	7 (2.3)	0	0	0	0	16 (11.3)	0	0	0	0
19F†	53 (17.4)	19 (6.9)	14 (3.8)	7 (2.2)	6 (3.2)	16 (11.3)	7 (13.2)	1 (2.0)	0	0
23F†	37 (12.1)	8 (2.9)	2 (0.5)	0	0	8 (5.6)	0	0	0	0
Total	189 (63.0)	33 (12.0)	17 (4.6)	7 (2.2)	6 (3.2)	121 (85.2)	11 (20.8)	1 (2.0)	0	0
Additional serotypes in PCV13										
1†	2 (0.7)	1 (0.4)	0	0	0	1 (0.7)	1 (1.9)	1 (2.0)	0	0
3†	14 (4.6)	22 (8.0)	14 (3.8)	13 (4.0)	0	0	1 (1.9)	3 (6.1)	2 (4.9)	0
5†	0	0	0	0	0	0	0	1 (2.0)	0	0
6A	24 (7.9)	16 (5.8)	5 (1.4)	1 (0.3)	1 (0.5)	11 (7.8)	3 (5.7)	0	0	0
7F†	1 (0.3)	2 (0.7)	6 (1.6)	2 (0.6)	0	1 (0.7)	0	8 (16.3)	2 (4.9)	0
19A†	11 (3.6)	68 (24.7)	135 (36.7)	107 (32.8)	94 (50.3)	1 (0.7)	15 (28.3)	18 (36.7)	18 (43.9)	13 (59.1)
Total	52 (17.3)	109 (39.6)	160 (43.5)	123 (37.7)	95 (50.8)	14 (9.9)	20 (37.7)	31 (63.3)	22 (53.7)	13 (59.1)
PCV-related										
6C	2 (0.7)	2 (0.7)	28 (7.6)	29 (8.9)	21 (11.2)	0	0	1 (2.0)	3 (7.3)	2 (9.1)
9N	4 (1.3)	1 (0.4)	2 (0.5)	2 (0.6)	0	0	0	0	0	0
23A	0	8 (2.9)	17 (4.6)	10 (3.1)	5 (2.7)	0	1 (1.9)	1 (2.0)	0	0
23B	0	4 (1.5)	14 (3.8)	9 (2.8)	1 (0.5)	0	0	0	2 (4.9)	2 (9.1)
Other	4 (1.3)‡	3 (1.1)§	5 (1.4)¶	1 (0.3)#	0	1 (0.7)‡	1 (1.9)§	0	0	0
Total	10 (3.3)	18 (6.5)	66 (17.9)	51 (15.6)	27 (14.4)	1 (0.7)	2 (3.8)	2 (4.1)	5 (12.2)	4 (18.2)
Non-PCV										
11A†	3 (1.0)	15 (5.5)	12 (3.3)	14 (4.3)	4 (2.1)	0	1 (1.9)	0	0	0
15A	3 (1.0)	9 (3.3)	13 (3.5)	2 (0.6)	0	0	1 (1.9)	3 (6.1)	1 (2.4)	1 (4.5)
15B†	3 (1.0)	10 (3.6)	14 (3.8)	20 (6.1)	11 (5.9)	1 (0.7)	2 (3.8)	0	2 (4.9)	0
15C	2 (0.7)	7 (2.6)	11 (3.0)	14 (4.3)	7 (3.7)	1 (0.7)	2 (3.8)	2 (4.1)	3 (7.3)	1 (4.5)
21	3 (1.0)	1 (0.4)	9 (2.5)	9 (2.8)	2 (1.1)	0	0	0	0	0
22F†	2 (0.7)	12 (4.4)	7 (1.9)	18 (5.5)	0	1 (0.7)	2 (3.8)	3 (6.1)	2 (4.9)	0
35B	6 (2.0)	23 (8.4)	20 (5.4)	36 (11.0)	35 (18.7)	0	1 (1.9)	0	3 (7.3)	3 (13.6)
Other	15 (5.0)**	36 (13.1)††	35 (9.5)‡‡	26 (8.0)§§	0	2 (1.4)**	11 (20.8)††	7 (14.3)‡‡	3 (7.3)§§	0
NT	12 (3.9)	2 (0.7)	4 (1.1)	6 (1.8)	0	1 (0.7)	0	0	0	0
Total	49 (16.3)	115 (41.8)	125 (34.0)	145 (44.5)	59 (31.6)	6 (4.2)	20 (37.7)	15 (30.6)	14 (34.1)	5 (22.7)
All	300	275	368	326	187	142	53	49	41	22
% Pen NS	50.2	48.0	59.2	57.4	NA	26.1	43.4	40.8	53.7	NA

*Values are no. (%) isolates unless otherwise indicate. PCV7, pneumococcal conjugate 7-valent vaccine; PCV13, pneumococcal conjugate 13-valent vaccine; Pen NS, penicillin nonsusceptible (MIC ≥0.12 μg/mL); NT, nontypeable; NA, not applicable; non, noninvasive; inv, invasive.
†Serotypes in 23-valent polysaccharide pneumococcal vaccine.
‡Other low prevalence serotypes (no. isolates): in 1999–00, serotype 9A (1 non; 1 inv), 18B (3 non).
§In 2004–2005, serotype 9A (1 non), 18A (1 non), 18B (1 non), 19B (1 inv).
¶In 2008–2009, serotype 7C (5 non).
#In 2010–11, serotype 7B (1 non).
**In 1999–2000, serotype 10A (3 non), 12F (1 inv), 13 (1 non), 16A (1 non), 17F (1non), 25A (2 non, 1 inv), 29 (1 non), 33F (2 non), 33A (1 non), 35F (2 non), 45 (1 non).
††In 2004–2005, serotype 10A (1 non), 12F (1 non, 1 inv), 16F (3 non, 2 inv), 16A (1 non), 20 (1 non, 1 inv), 25A (1 inv), 29 (6 non), 31 (5 non, 1 inv), 33F (4 non), 33A (1 non, 2 inv), 34 (1 non), 35F (5 non, 1 inv), 35A (4 non), 38 (1 non, 2 inv), 39 (1 non), 48 (1 non).
‡‡In 2008–2009, serotype 8 (1 non), 10A (4 non), 12F (1 inv), 16F (5 non), 17F (4 non, 2 inv), 20 (1 non), 22A (1 non), 25A (1 non), 29 (1 non, 1 inv), 31 (2 non), 33F (2 non), 33A (2 non, 1 inv), 33B (1 non), 34 (3 non, 1 inv), 35F (5 non, 1 inv), 35C (1 non), 42 (1 non).
§§In 2010–2011, serotype 10A (5 non), 16F (5 non), 17F (3 non), 24A (1 non), 31 (2 non), 33F (2 non, 1 inv), 33A (1 non, 2 inv), 34 (2 non), 35F (4 non), 45 (1 non).

Although serotype 6A is included in PCV13, steady decreases in the number of 6A and 6B isolates observed since 1999–2000 suggest cross-reactivity of the 6B PCV7 component against 6A strains. The newly recognized serotype 6C strains increased from 0.8% during 1999–2000 to 8.5% of all isolates during 2010–2011, and a similar change (from 0.5% to 8.7%) was observed in children ≤5 years of age. Intermediate resistance to penicillin was noted in 50% of the serotype 6C isolates obtained from all age groups and in 72% of 6C strains recovered from the youngest patient age group. Opsonophagocytic killing studies comparing responses of serum from PCV7 and PCV13 recipients to serotype 6C showed minimal response with PCV7, but a strong response was elicited by PCV13 (*31*). Responses

to serotype 6A and 6B were strong for immune serum samples from PCV7 and PCV13 recipients (*31*). In the current study, lack of a major increase in the frequency of isolates with serotype 6C during 2010–2011 (8.5%) compared with 2008–2009 (7.3%; p = 0.17) suggests PCV13 cross-reactivity to serotype 6C.

The trend of a relative increase in serotype 35B strains observed in this study is likely to continue because it is not included in PCV13. Serotype 35B isolates were more commonly recovered from children ≤5 years of age and from noninvasive specimens. Most (83%) of the 35B isolates obtained in 2010–2011 were PNSP. CDC reported that 51% of serotype 35B strains causing invasive disease during 1995–2001 were from older patients (>60 years of age) and 69% were PNSP (*32*).

A limitation of this study is the lack of incidence data. Only relative changes among serotypes causing disease are documented. The proximity in time of 2010–2011 data to PCV13 introduction limited our assessment of the effect of this vaccine.

On the basis of this 2010–2011 surveillance data, PCV13 could provide coverage for 48% of PNSP and 39% of all isolates causing disease. Continued monitoring of pneumococcal serotypes causing invasive and noninvasive disease will be crucial for assessing the full effect of PCV13.

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Influence of Pneumococcal Vaccines and Respiratory Syncytial Virus on Alveolar Pneumonia, Israel

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Postlicensure surveillance of pneumonia incidence can be used to estimate whether pneumococcal conjugate vaccines (PCVs) affect incidence. We used Poisson regression models that control for baseline seasonality to determine the impact of PCVs and the possible effects of variations in virus activity in Israel on these surveillance estimates. PCV was associated with significant declines in radiologically confirmed alveolar pneumonia (RCAP) among patients <6 months, 6–17 months, and 18–35 months of age (–31% [95% CI –51% to –15%], –41% [95% CI –52 to –32%], and –34% [95% CI –42% to –25%], respectively). Respiratory syncytial virus (RSV) activity was associated with strong increases in RCAP incidence, with up to 44% of cases attributable to RSV among infants <6 months of age and lower but significant impacts in older children. Seasonal variations, particularly in RSV activity, masked the impact of 7-valent PCVs, especially for young children in the first 2 years after vaccine introduction.

Streptococcus pneumoniae is a major cause of pneumonia worldwide, but in only a small fraction of severe cases are bacteria detectable in blood or cerebrospinal fluid (*1*). Because of the limitations of the diagnostic tools, identifying pneumococcal pneumonia is difficult and insensitive. Thus, using an endpoint of radiologically confirmed alveolar pneumonia (RCAP) can provide a more sensitive, but less specific, diagnosis of bacterial pneumonia that can be used to monitor the impact of pneumococcal conjugate vaccines (PCVs) on disease incidence (*2*,*3*).

The effect of vaccination on nonbacteremic pneumonia can be determined in the context of randomized control trials (*4*), in case–control studies (*5*,*6*), or by monitoring changes in the incidence of disease through routine surveillance (*7*). Of these options, surveillance data are the most readily available and give the most realistic estimate of vaccine effect, but they also are subject to biases, including secular trends and changes in detection or reporting (*8*,*9*). In the monitoring of nonbacteremic pneumonia incidence in children, a major source of variation might be year-to-year fluctuations in virus activity, which could influence the baseline and the post-PCV estimates of incidence (*7*,*10*).

Southern Israel provides a unique setting for evaluating the influence of PCVs on the incidence of pneumonia in children and the contributing role of respiratory viruses toward these estimates. The Jewish and Bedouin populations in this region inhabit the same geographic area but have limited social contact and generally differ in terms of socioeconomic status and illness patterns (*11*,*12*). Reflecting this diversity, there was little uptake of 7-valent PCV (PVC7) by the Bedouin population before the vaccine was introduced into the national immunization program in 2009 but moderate use among Jewish children on the private market. Since 2009, vaccine coverage has increased rapidly in both populations.

We sought to quantify the effect of PCV vaccination on RCAP incidence in southern Isreal. We took advantage of ongoing prospective studies of pediatric RCAP incidence and virus prevalence in children (*13*,*14*) that were conducted in the only hospital in southern Israel, which covers 95% of the population. We used Poisson regression models to estimate and control for the effects of seasonality and

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respiratory syncytial virus (RSV) and influenza activity on RCAP incidence and to determine the decline in incidence associated with increased uptake of PCVs.

Methods

Data Sources

RCAP cases were diagnosed at the Soroka University Medical Center, the only medical center in southern Israel, as described (*13*,*14*). The total population of children <3 years of age in the region in 2009 was ≈45,000, of whom 23,439 were Jewish children and 21,596 were Bedouin children. A case was defined as chest radiographic evidence of alveolar pneumonia in a patient <3 years of age who was seen in the pediatric emergency department or admitted to the hospital and in whom RCAP had not been diagnosed within the previous 28 days. Chest radiographs were analyzed according to the World Health Organization Standardization of Interpretation of Chest Radiographs Working Group (*15*). All readings were collected daily and read by 2 pediatric infectious diseases specialists (D.G. and R.D.) and independently confirmed by a pediatric radiologist, as described (*13*,*14*). The cases were aggregated into weekly time series for July 2004–June 2012 and stratified by ethnicity (Jewish vs. Bedouin) and by age group (<6 months, 6–17 months, and 18–35 months). Population size for each stratum was estimated on the basis of the number of births among the Jewish and non-Jewish populations in each calendar year in Beer-Sheva (*16*).

Uptake of PCV7 and 13-valent PCV (PCV13) was determined from an ongoing hospital survey starting in July 2009, when Israel's national immunization program began covering PCV7. We enrolled the first 4 Jewish children and the first 4 Bedouin children <5 years of age seen on each working day at the pediatric emergency department of the Soroka Medical Center whose parents consented. The children's vaccinating centers provided data on birth date, vaccination date, and whether the vaccine was PCV7 or PCV13. More than 95% of vaccinating centers responded to the questionnaires. During July 2009–April 2012, a total of 2,555 Jewish children and 2,666 Bedouin children were enrolled. Before the survey, no data were available on vaccine uptake. Health maintenance organizations (HMOs) began covering PCV7 in summer 2008; uptake among Jewish children was moderate (up to 25%) but was lower among Bedouin children. For the period before mid-2008, we assumed no vaccine uptake in either ethnic group. To fill in the values from the third quarter of 2008 through the second quarter of 2009, we assumed that coverage increased linearly from 0 in 2008 to observed vaccine uptake in 2009. We calculated the uptake in each quarter of the year and assigned this coverage to all weeks in that period.

Starting in July 2004, nasopharyngeal wash specimens were obtained from children during working days for virus testing. The decision to obtain nasopharyngeal wash specimens was made by the treating physicians and driven by clinical judgment. Specimens were obtained mostly from children admitted to the hospital and were processed in the virology laboratory within 6 hours. All samples were tested for RSV; influenza viruses A and B; and parainfluenza viruses 1, 2, and 3 by using either PCR or direct immunofluorescence antibody testing and commercial monoclonal antibodies (Chemicon, Temecula, CA, USA) supplemented by tissue culture (*17*,*18*). Weekly RSV and influenza counts were divided by the total number of swabs tested in that July–June year to adjust for biases in testing. These calculations were performed separately for Jewish and Bedouin children.

As an alternate measure of RSV activity, we used the weekly incidence of bronchiolitis among Jewish or Bedouin children. For this purpose, we retrieved from the hospital computer database all hospital visits (inpatients and outpatients) of children <3 years old for whom the final diagnoses included "bronchiolitis." Monthly climate data on temperature, humidity, precipitation, and wind speed were obtained from the Central Bureau of Statistics (*16*) for the Beer-Sheva West meteorologic station.

Estimating Vaccine Impacts

Unadjusted incidence rate ratios were calculated by determining the incidence in a given year (July–June) or given season (November–April or May–October) and dividing it by the mean incidence of the corresponding prevaccine period. To estimate the effects of RSV, influenza, and vaccine uptake on RCAP incidence, we fit a Poisson regression (log-link) model using PROC GENMOD in SAS version 9.2 (SAS Institute, Cary, NC, USA). The outcome variable was the weekly incidence of RCAP. The predictor variables were weekly activity of influenza and RSV among Jewish and Bedouin children (as described above), an indicator variable for ethnicity; sine and cosine terms that had frequencies of 52.25 and 26.125 weeks and varied between ethnic groups; and a binary variable that indicated whether the PCV vaccination program in the Jewish or Bedouin population had reached maturation. The vaccination program was considered to be mature when at least 85% of the Jewish or Bedouin populations 6–17 months of age had received at least 2 doses of any PCV (85% was chosen to indicate an immunization program with stable rates [Figure 1]). We also considered using vaccine uptake as a continuous variable in the model, but the relationship between vaccine uptake and incidence appeared to have a threshold effect, as might be expected given the role of herd immunity (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/7/12-1625-Techapp1.pdf).

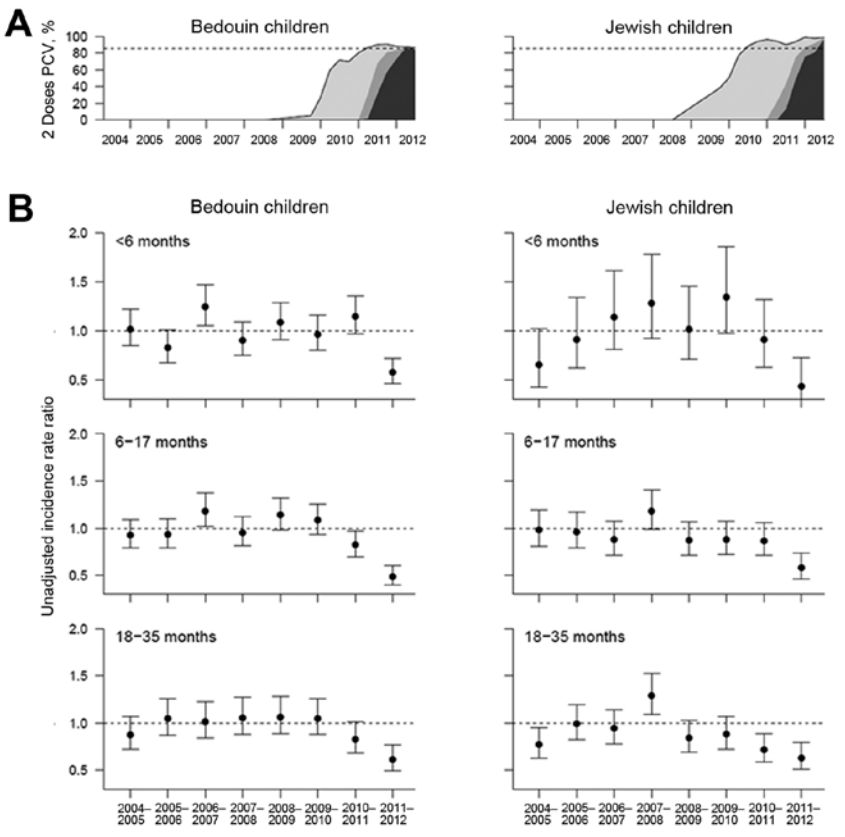

Figure 1. PCV uptake and decline in RCAP incidence 2004–05 through 2011–12, southern Israel. A) Uptake of >2 PCV doses among Bedouin and Jewish children 6–17 months of age. Black line indicates overall uptake; shaded areas show the proportion receiving >2 doses of PCV7 (light gray), >2 doses of PCV13 (dark gray), or >1 doses of PCV7 and 1 dose of PCV13 (medium gray). Dotted line indicates 85% uptake of any PCV. B) Unadjusted incidence rate ratio for RCAP comparing the incidence in each July–June year with the average for 2004–05 through 2007–08. RCAP, radiologically confirmed alveolar pneumonia; PCV, pneumococcal conjugate vaccine; PCV7, 7-valent PCV; PCV13, 13-valent PCV. Error bars indicate 95% CIs.

This model assumes that the effects of the viruses and vaccination are the same for both ethnic groups but enables the seasonal baseline to vary in timing and intensity between ethnic groups. Periodic variables were selected on the basis of Akaike Information Criteria in preliminary fixed-effects analyses, and other variables were included if they were significant and improved the Akaike Information Criteria score. Interaction terms between ethnicity and the virus variables or vaccine uptake variable were tested but not included in the final model. The distribution of the deviance residuals was approximately normal for all strata except for the <6-month-old Jewish children (where the data were sparse).

We determined 95% CIs for the parameters using an 8-week moving-block bootstrap (n = 1,000) to account for autocorrelation in the time series (*19*,*20*). The incidence rate ratio associated with high vaccine uptake in each age group was estimated by taking the exponent of the regression coefficient for the vaccine uptake variable. The percentage change was estimated as 100 × (incidence rate ratio – 1); a negative percentage indicates a decline in incidence. The effects of influenza and RSV were calculated by fitting the model and then obtaining predicted values by substituting either the observed value for RSV or influenza or 0 into the equation. The difference between the estimates constituted the virus-attributed incidence, and the attributable percentage was the virus-attributed incidence divided by total estimated incidence. To validate the approach, we fit an alternative model in which the 2011–12 season was excluded or a model that excluded RSV and made predictions for the 2011–12 season. We also tested for variations in climate across the study period, such as monthly temperature and precipitation, that might be associated with the observed declines in RCAP incidence.

Results

From 2004–05 through 2011–12, a total of 2,246 RCAP cases occurred among Jewish children <3 years of age and 3,690 cases occurred among Bedouin children <3 years of age (Table 1). Incidence was highest among the Bedouin children, and age at infection varied substantially between Jewish and Bedouin children (Table 1). For Jewish children, incidence peaked among 6–17-month-olds; for Bedouin children, among <6-month-olds. The incidence of RCAP (Table 1; Figure 1) and respiratory viruses (online Technical Appendix Figure 2) varied substantially between seasons.

Changes in Alveolar Pneumonia Incidence, 2004–2012

We first evaluated whether the incidence of RCAP changed after PCV was introduced into the national immunization program. RCAP incidence decreased significantly in 2011–12 compared with the prevaccine era,

Table 1. RCAP incidence, southern Israel, 2004–2012*

Ethnicity; age, mo.	RCAP, no. cases (incidence†)								
	Overall	2004–05	2005–06	2006–07	2007–08	2008–09	2009–10	2010–11	2011–12
Jewish									
<6	285 (9.1)	23 (6.2)	32 (8.7)	41 (10.8)	48 (12.4)	38 (9.6)	51 (12.8)	35 (8.6)	17 (4.1)
6–17	958 (15.6)	128 (16.9)	123 (16.6)	112 (15.2)	157 (20.7)	117 (15.1)	120 (15.2)	120 (15.0)	81 (10.1)
17–35	1,003 (11.0)	106 (9.6)	140 (12.3)	130 (11.7)	181 (16.3)	119 (10.5)	127 (10.9)	106 (9.0)	94 (7.9)
Bedouin									
<6	1,147 (39.9)	147 (41.9)	116 (33.9)	177 (51.1)	135 (37.9)	162 (44.5)	146 (39.6)	175 (47.0)	89 (23.7)
6–17	1,486 (26.0)	186 (25.7)	181 (25.8)	223 (32.6)	186 (26.9)	224 (31.4)	218 (29.9)	168 (22.8)	100 (13.5)
17–35	1,057 (12.4)	123 (11.5)	149 (13.7)	140 (13.3)	145 (14.1)	145 (14.0)	147 (13.8)	119 (10.9)	89 (8.1)

*RCAP, radiologically confirmed alveolar pneumonia.
†Number of cases/1,000 children/year.

by ≈49%, ≈46%, and ≈37% among Jewish and Bedouin children <6 months, 6–17 months, and 18–35 months of age, respectively (Figure 1, panel B). For the preceding year, 2010–11, RCAP incidence declined modestly among 18–35-month-old Jewish children (28%, 95% CI 11.0%–41%), Bedouin children overall (17%, 95% CI –1% to 32%), and 6–17-month-old Bedouin children (17%, 95% CI 2%–30%). However, RCAP incidence did not decline among Jewish children <6 months or 6–17 months of age, despite high uptake of the vaccine.

We also compared changes in incidence during winter months with changes in incidence during summer months, when there is little respiratory virus activity. RCAP incidence declined significantly in the summer of 2010 compared with the prevaccine era in the Jewish population but was similar to baseline levels during the following winter (online Technical Appendix Figure 2, panel B). Among Bedouin children, where vaccine uptake was slower (Figure 1, panel A), RCAP incidence did not decline significantly until summer 2011.

Association between RCAP Incidence and Activity of Respiratory Viruses

We next considered whether year-to-year variations in virus activity (online Technical Appendix Figure 3) might influence estimates of vaccine impact and perhaps mask the effect of the vaccine during the first few years or lead to overestimation of vaccine effect during the weak RSV and influenza season of 2011–12. To evaluate the relationship between respiratory virus activity and RCAP incidence, we fit a regression model that accounts for virus activity, vaccine uptake, and baseline seasonal variations. RSV and influenza were associated with significant increases in RCAP incidence; RSV was associated with an especially strong increase. A total of 44% (95% CI 36%–52%), 21% (95% CI 15%–25%), and 16% (95% CI 11%–20%) of RCAP cases were attributed to RSV among children <6 months, 6–18 months, and 18–36 months of age, respectively (online Technical Appendix Figure 4). Influenza was more modestly associated with RCAP incidence, with 1% (95% CI –6% to 6%), 4% (95% CI 1%–6%), and 2% (95% CI 1%–4%) of disease attributed to influenza among children <6 months, 6–18 months, and 18–36 months of age, respectively (online Technical Appendix Figure 4).

When we controlled for virus activity, vaccination (>85% uptake) was associated with a significant decline in RCAP incidence of –31% (95% CI –51% to –15%), –41% (95% CI –52 to –32%), and –34% (95% CI –42% to –25%) among <6-month-old, 6–18-month-old, and 18–35-month-old children, respectively. Results from a model in which bronchiolitis hospitalizations, rather than RSV positivity, was used as a predictor gave similar results through the end of 2011 (data not shown). We also considered the possibility that the low RCAP seasons might be associated with anomalous climatic conditions but did not detect any significant differences in monthly temperature, humidity, precipitation, or wind across the study period that might explain the observed patterns; these variables were not included in the final model.

To evaluate the sensitivity of our results to the RCAP incidence in 2011–12, we refit the model to data from 2003–04 through 2010–11 and extrapolated the change in incidence for 2011–12. The model accurately predicted the observed change in RCAP incidence in 2011–12 for all age groups (Figure 2).

Importance of Including RSV and Seasonal Variables in the Analyses

As a sensitivity analysis, we tested whether inclusion of RSV in the model influenced the estimates of vaccine impact. When fitting the model to the entire dataset (2004–05 through 2011–12), removing RSV from the model had little or no effect on estimates of vaccine impact in any of the age groups (Table 2). However, when we fit the model to data only from 2004–05 through 2010–11 (including the first 2 years of PCV7 use) and then evaluate the impact of excluding RSV from the model, we found that RSV substantially influenced the estimates in children <6 months old (Table 2). Removing RSV from the model modestly affected the estimate of vaccine impact among the 6–17-month-old children and had no detectable on the estimates among the 18–35-month-old children (Table 2). This finding suggests that RSV masked the impact of

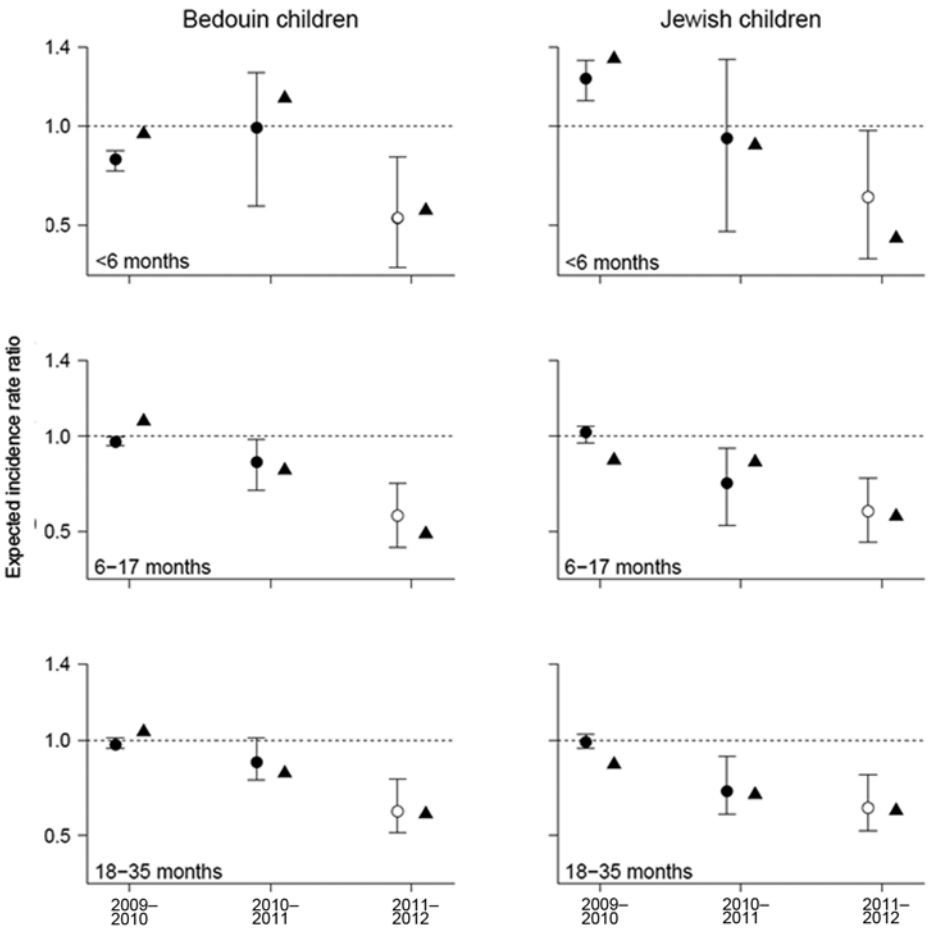

Figure 2. Observed (triangles) and expected (closed circles) change in RCAP incidence for each year compared with the 2004–05 through 2007–08 average, expressed as incidence rate ratios, southern Israel. Expected values were determined from a model fit to data from 2004–05 through 2010–11, with the 2011–12 values (open circles) extrapolated based on the observed virus activity. Error bars indicate 95% CIs.

vaccination on RCAP incidence in young infants in the first full year after vaccine introduction.

We also fit a simple model that did not include variables for virus activity or seasonal (harmonic) terms. Again, when the model includes data through the 2011–12 season, the omission of these variables had little impact on the estimates of vaccine impact (Table 2). However, when the model was fit just to data through the 2010–11 season, the estimate of vaccine impact was closer to 0, especially among younger children.

Discussion

We have demonstrated that introduction of PCVs into the national immunization program in Israel was associated with a significant decline in the incidence of RCAP among both Jewish and Bedouin children. RSV activity and RCAP incidence were strongly associated, and controlling for such variation, along with regular seasonal fluctuations, improved our estimates of vaccine impact in young children, especially in the immediate post–PCV implementation period. These analyses highlight a potential bias that can influence estimates of vaccine-associated declines, particularly with short surveillance follow-up periods.

Most alveolar pneumonia is believed to have a bacterial etiology, either alone or in combination with a virus (*14*,*21*–*23*). A recent study from this population in Israel demonstrated that 37% of children <1 year old and 14.7% of children 1–2 years old who had RCAP had detectable RSV (*2*). The results of our analysis also support a strong association between RSV activity and RCAP incidence, particularly in children <6 months of age. RSV could potentially increase the risk of developing secondary bacterial infections, but using these data, we were unable to determine whether the RSV-attributable RCAP cases resulted from RSV infections alone or from viral–bacterial co-infections.

A previous study in South Africa found that a 9-valent PCV was associated with a vaccine efficacy against radiologically confirmed pneumonia of ≈20% among HIV-uninfected persons (*24*). The higher impact of PCV estimated in our analyses could be because of the age distribution of case-patients and the more specific diagnosis of alveolar pneumonia, most of which is most

Table 2. Impact of pneumococcal conjugate vaccine from different models, southern Israel*

Age group, mo.	Fit to 2004–05 through 2011–12			Fit to 2004–05 through 2010–11 (excluding 2011–12)		
	Full model	Model without RSV†	Unadjusted‡	Full model	Model without RSV†	Unadjusted‡
<6	−31.5	−30.7	−20.0	−27.9	−5.2	**+22.3**
	(−50.6 to −14.5)	(−57.2 to 9.7)	(−70.2 to 76.5)	(−62.9 to 9.2)	(−68.5 to 83.9)	**(−81.5 to +106.0)**
6–17	−40.5	−39.6	−36.0	−36.3	−29.9	−20.0
	(−52.1 to −31.5)	(−52.7 to −26.0)	(−63.4 to 4.6)	(−53.6 to −19.7)	(−53.7 to −7.8)	(−69.5 to +44.0)
17–35	−33.6	−33.0	−31.3	−33.3	−29.1	−25.4
	(−41.5 to −25)	(−40.9 tp −23.1)	(−47.8 to −8.4)	(−45.0 to −16.0)	(−44.2 to −10.8)	(−55.0 to +11.1)

*Vaccine impact is the estimated percentage change in disease incidence associated with pneumococcal conjugate vaccine use. RSV, respiratory syncytial virus.
†Model fit with all predictors described in the methods section except for RSV activity.
‡Model fit with predictors for vaccine uptake and ethnicity only.

likely bacterial. Additionally, the herd immunity effect will be stronger after licensure than in a randomized control trial (*25*). Vaccine effectiveness against pneumonia also will depend on which pathogens are prevalent in a given region and the etiologic fraction of each. Finally, our study population had high uptake of PCV13, which targets serotypes 1, 5, 7F, and 19A—types associated with RCAP in young children in our region (*13*).

The measured vaccine impacts presented here resulted both from direct and indirect (herd immunity) effects. The indirect benefit of pneumococcal vaccination on unvaccinated age groups has been well documented (*7*), and we did not try to disentangle the relative contributions of direct or indirect protection in our analyses.

Our results assume a relationship between RSV incidence and alveolar pneumonia that remained consistent across the entire study period. Use of PCVs can influence viral incidence resulting from bacterial–viral interactions (*26*), which possibly could bias our results. Although we did not detect such a trend in the RSV data in this population, if such a bias existed, then decreases associated with the vaccine might be attributed instead to the virus decline, and the estimated impact of the vaccine would be attenuated. The estimated effect of influenza was smaller than for RSV but might have been more pronounced if we had been able to estimate separate effects for seasons during which influenza A(H3N2) is severe and seasons in which influenza A(H1N1) and influenza B are comparatively mild. Examining the impact of PCVs on the incidence or severity of viral pneumonias would be a promising area for future research.

The measurement of vaccine uptake used here is based on a hospital survey, which could be biased if health care use differs by ethnicity or economic status. This hospital has the only emergency department in the region, and it serves all Jewish and Bedouin children in the region. The immunization survey also measured diphtheria–pertussis–tetanus vaccination and found >90% uptake among both Jewish and Bedouin children. Because vaccine uptake was included as a threshold variable in the model, the results are relatively insensitive to any potential biases in the uptake data.

We considered an alternative model with a continuous vaccine uptake variable. This model gave comparable estimates of vaccine impact in 2010 for the 18–35-month age group (−30% for continuous vs. −34% for binary). The estimated decline was smaller when we used the continuous vaccine uptake variable for children <6 months (−18% vs. −31%) and 6–17 months of age (−32 vs. −41%).

Focusing on RCAP as an outcome has advantages and disadvantages. The diagnosis of RCAP in this study follows a well-defined protocol (*14*) and has undergone quality control validations. However, focusing just on RCAP can underestimate the total impact of pneumococcal vaccination (*2*). Therefore, our estimates, although based on high-quality data, possibly underestimate the total impact of the vaccine.

This model does not explicitly estimate the contribution of pneumococcal disease to RCAP incidence. Rather, we assume that any changes in the postvaccine period are attributable to the vaccine or to variations in RSV or influenza activity. If additional factors not accounted for by the model caused a decline in RCAP, we would overestimate the impact of the vaccine. Likewise, we assume that the seasonal terms (harmonics) capture weekly variations not attributable to virus activity. If these seasonal terms do not fit the data well, they would overestimate the effects of the viruses.

Given our analytical methods, we cannot definitively say that RSV is an independent cause of alveolar pneumonia, which is typically considered to have a bacterial cause. Further work with case–control studies or other designs is needed to be done to establish a causal link. Likewise, because our study was an ecologic study, we cannot definitively attribute any decreases to the vaccine. Longer-term surveillance is needed to confirm that the rates remain lower than the historical baseline, taking into account virus activity and other possible confounding factors.

In the absence of bacteriologic data from these patients, fully determining the relative importance of PCV7 and PCV13 is impossible, especially given the short observation period before PCV13 was adopted. The observed incidence rate for each quarter suggests a substantial drop in RCAP incidence before PCV13 uptake

was high (online Technical Appendix Figure 1). On the basis of invasive pneumococcal disease (IPD) data from Israel, in children <5 years of age, PCV7 serotypes were reduced by 81% after PCV implementation, with an overall reduction in IPD incidence of 43% by the end of 2010 (*27*). The remaining PCV13 serotypes accounting for 72% of the residual disease (*27*). As a result, PCV13 introduction could account for a considerable additional drop in IPD incidence in 2010–11 and 2011–12 if RCAP followed a pattern similar to IPD. Furthermore, some of the additional serotypes in PCV13 have high case/carrier ratios and therefore might cause a large decrease in disease incidence (*13*).

We have shown that the incidence of RCAP significantly declined after PCV was introduced and that statistical models accounting for virus activity can help to improve interpretation of these data. Such analyses can be used in conjunctions with other methods, such as case–control studies, active population-based surveillance, and carriage studies, to evaluate the impact of vaccination.

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Avian Metapneumovirus Subgroup C Infection in Chickens, China

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Avian metapneumovirus causes acute respiratory tract infection and reductions in egg production in various avian species. We isolated and characterized an increasingly prevalent avian metapneumovirus subgroup C strain from meat-type commercial chickens with severe respiratory signs in China. Culling of infected flocks could lead to economic consequences.

Avian metapneumovirus (aMPV), in the subfamily *Pneumovirinae* of the family *Paramyxoviridae*, is associated with acute respiratory tract infection as well as reductions in egg production in turkeys, chickens, and ducks (*1*). aMPV contains a nonsegmented, single-stranded, negative-sense RNA genome of approximately 13 kb in length, organized as 3′-leader-N-P-M-F-M2-SH-G-L-trailer-5′ (*2*). Based on genetic and antigenic properties, aMPV can be classified into 4 subgroups: A, B, C, and D. After its first detection in South Africa in 1978 (*3*), different subgroups of aMPV, mainly A or B, were reported in Europe, Asia, and some other parts of the world in turkeys and chickens. Subgroup C aMPV was first reported in turkeys in the United States in 1996 (*4*) and subsequently isolated from farmed ducks in France (*5*) and pheasants in South Korea (*6*), as well as some wild birds (e.g., American coots, American crows, Canada geese, cattle egrets, and sparrows). These isolates are different both genetically and antigenically from subgroups A and B. Here, we report the isolation and characterization of aMPV subgroup C (aMPV-C) in chickens in China.

The Study

During February–April 2012, severe respiratory infection in chickens was observed on some local meat-type commercial chicken farms in southeastern China. The affected chickens ranged in age from 20 to >60 days. Clinical signs were an acute and severe respiratory disease characterized by nasal and ocular discharge, foamy conjunctivitis, inflamed eyes, facial congestion, tracheal rales, swollen infraorbital sinuses, and visible yellow to white caseous discharge from the nasal sinuses or trachea. Illness rates were 30%–80% in different chicken flocks, but mortality rates were often <15%.

On a farm where yellow-feathered chickens were raised, we collected 5 nasal turbinate samples from birds that were 48 days old for further laboratory testing. These nasal turbinate samples were resuspended in minimum essential medium, and total RNA was extracted for detection of aMPV by using reverse transcription PCR (RT-PCR) subtyping as described (*7*). All 5 samples (100%) were positive for aMPV-C. In addition, the nasal turbinate samples were inoculated into Vero cell lines for 5 blind passages, and aMPV viral antigens were further detected by immunofluorescent assay by using a rabbit polyclonal antibody raised against a polypeptide located in the N protein of all aMPV subgroups (*8*). We also used PCR or RT-PCR to detect potentially related viruses (avian influenza virus subtypes H5 and H9, Newcastle disease virus, infectious laryngotracheitis virus, and infectious bronchitis virus) and used RNA or DNA isolated from homologous virus stocks as positive controls. The extracted samples did not react with the respective virus-specific primers, further indicating that aMPV-C may be a major pathogen in these affected chickens.

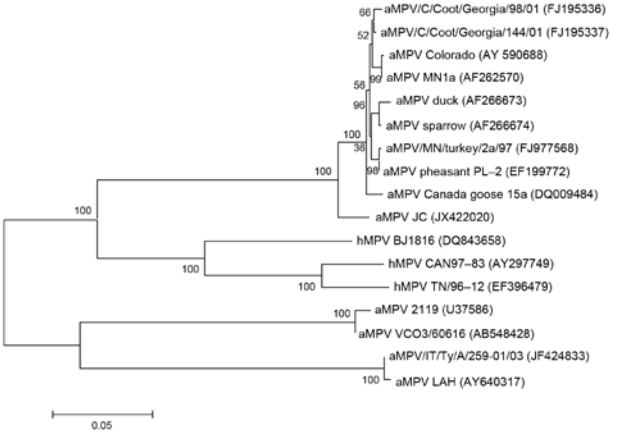

Figure 1. Genetic relatedness between the matrix (M) protein gene of members of avian metapneumoviruses (aMPV) and human metapneumovirus (hMPV). Phylogenetic tree was constructed on the basis of the neighbor-joining clustering method by using MEGA 5.10 software (www.megasoftware.net). Bootstrap values (based on 500 replicates) are indicated at each branching point. Reference strains obtained from GenBank are indicated. The M sequence of the isolate JC used in the phylogenetic analysis has been deposited in GenBank under accession no. JX422020. Scale bar indicates estimated phylogenetic divergence.

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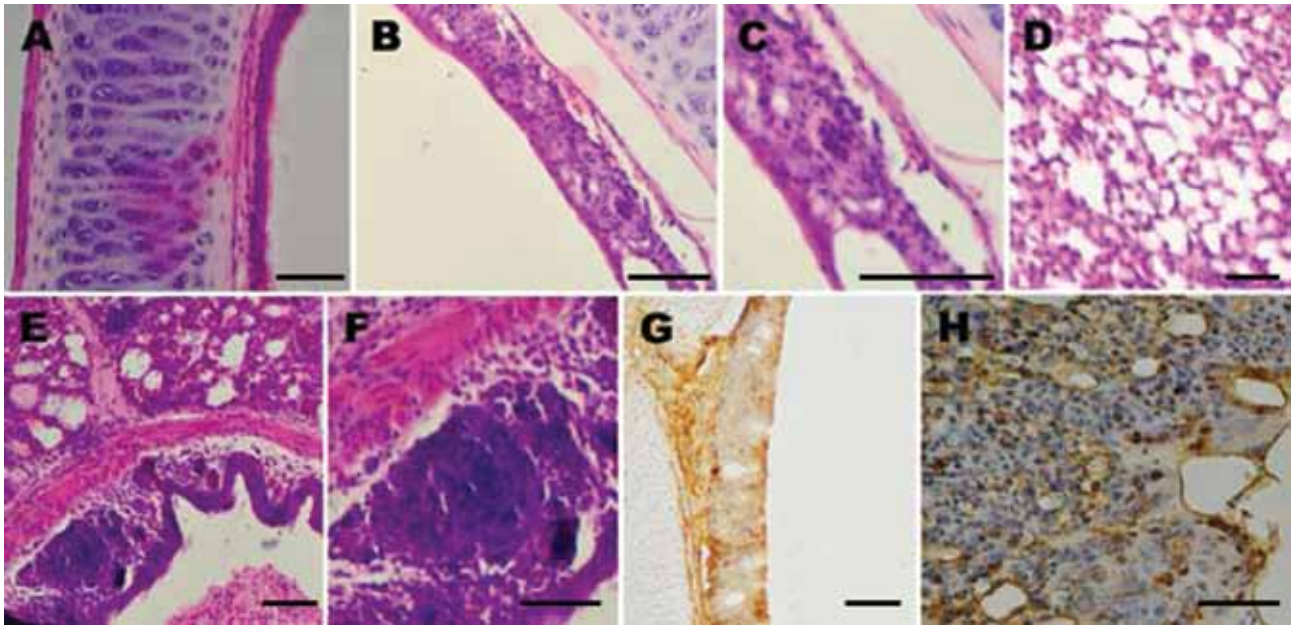

Figure 2. Histological appearance and immunohistochemical staining of respiratory tract samples collected from chickens before and after inoculation with avian metapneumovirus (aMPV) subgroup C, China. A) Trachea section from an uninoculated chicken shows intact ciliated epithelium. B) At 5 days' postinoculation, loss of cilia, architectural disruption, and infiltration of inflammatory cells were seen in most of the epithelium and submucosa of inoculated chickens. C) Same lymphoid cell infiltration in trachea as in panel B, showing large numbers of lymphocytes in the epithelium. D) Lung section from an uninoculated chicken shows no significant inflammation. E) At 5 days' postinoculation, inflammatory infiltration including lymphocytes, as well as scattered macrophages and heterophils were seen in most of the lungs of inoculated chickens. F) Same inflammatory infiltration in lung as in panel E, showing large numbers of lymphocytes in the bronchial submucosa of lung. G) Trachea tissue of aMPV-inoculated chicken shows many positive cells for aMPV antigen. H) Lung tissue of aMPV-inoculated chicken shows many positive cells for aMPV antigen. Scale bars indicate 80 μm.

The full-length sequences of matrix (M) protein gene from these nasal turbinate samples were further determined and were genetically identical. The M gene sequence from the aMPV-C isolate designated as strain JC was submitted to GenBank under accession no. JX422020. Phylogenetic and molecular evolutionary analyses were conducted by using MEGA 5.10 (www.megasoftware.net). This gene has a length of 765 nucleotides (nt) encoding 254 amino acids (aa), which shared 96.0%–96.7% nt identity with aMPV-C isolates from duck, turkey, pheasant, and wild bird samples, but 70.6%–71.7% nt identity with the aMPV subgroup A and B isolates.

The chicken isolate strain JC is more closely related to human metapneumovirus (hMPV) isolates (76.6%–78.5%) than other aMPV-C isolates (75.5%–77.8%). Notably, the chicken isolate JC showed the highest identity (78.5%) to hMPV strain BJ1816, which was isolated in China. In addition, there is higher identity (98.3%–99.0%) among other aMPV-C isolates than when compared to isolate JC (96.0%–96.7%). At the amino acid level, the M protein of isolate JC shared 98.0% aa identity with the duck isolate from France, 99.2%–99.6% aa identity with the turkey and pheasant isolates, and 99.2%–99.6% aa identity with the wild bird aMPV-C isolates but 76.9%–78.4% aa identity with the aMPV subgroup A and B isolates.

The phylogenetic tree of the nt sequences of aMPV representative subgroups as well as hMPV was constructed by using the neighbor-joining clustering method (Figure 1). The chicken aMPV strain JC formed 1 cluster with other aMPV-C viruses. Within the cluster, the isolate JC formed separate subclusters more similar to wild birds such as the Canada goose, suggesting that the aMPV-C might be derived from wild birds. However, the origin of the aMPV-C infection in chickens should be further studied.

We further determined the pathogenesis of the aMPV-C strain JC in specific-pathogen–free (SPF) chickens. A total of 30 two-week-old SPF chickens were randomly divided into 2 groups and placed in the animal housing units of the Institute of Animal Husbandry and Veterinary Medicine, Beijing Academy of Agriculture and Forestry Sciences. Handling of birds was in accordance with the Guidelines of Animal Care and Use Committee of the institute. One group was intranasally inoculated with the aMPV-C strain JC at a dose of $10^{4.25}$ by using tissue culture infectious dose 50 assay, whereas the other group was mock inoculated with 200 μL of Vero cell supernatant. Some of the inoculated chickens showed symptoms

such as nasal discharge, sinus swelling, and watery eyes at 3–7 days postinoculation (dpi). Nasal discharge and oropharyngeal swabs were collected at 3, 5, and 7 dpi, and viral replication was detected by using RT-PCR. All tracheae and lung samples were collected from 5 birds each time at 3, 5, and 7 days dpi for histopathologic examination and immunohistochemical staining by using standard procedures.

Histopathological findings were characterized by mild to severe inflammation in the tracheae and lungs (Figure 2), including disruption of the epithelial architecture, sloughing of epithelial cells, loss of ciliation, and infiltration of inflammatory cells. We documented hemorrhage, infiltration of lymphoid cells, and hyperplasia in the tracheal epithelium and propria lamina of the aMPV-C–infected chickens. Examination of the lungs showed peribronchial lymphoplasmocytic infiltrates, edematous thickening of the bronchial submucosa, and lymphoid cell hyperplasia. Diffuse mild expansion of the alveolar interstitium caused by mononuclear cell infiltrates and edema was also observed. Sloughed epithelial cells, heterophils, macrophages, and amorphous debris were visible in the bronchial lumens. Immunohistochemical staining by using rabbit antibody against a polypeptide located in aMPV N protein (*8*) revealed that viral antigen was detected in both morphologically normal and degenerated respiratory epithelial cells (Figure 2). In addition, mucus cells, basal cells, and luminal cellular debris that included sloughed epithelial cells and macrophages stained positive for aMPV antigen. Research data suggested that aMPV showed no good replication ability in chickens (*9*,*10*), and that clinical signs induced by aMPV alone are not apparent in chickens. In the present study, we found that the chicken aMPV-C isolate caused severe respiratory infection and pathological inflammatory lesions in chickens, indicating that the chicken aMPV-C isolate has more severe pathogenicity than other aMPV subgroup isolates for chickens. These observations are similar to those made with hMPV infection in animal models (*11*,*12*). However, the exact mechanism needs to be further studied.

Conclusions

We identified infection with subgroup C aMPV infection in local meat-type commercial chickens with variable severe respiratory signs in China. These findings show that aMPV-C viruses are a potential hazard for chickens, leading to ecological and economic issues. Therefore, more epidemiologic and molecular studies are needed to further assess economic losses caused by aMPV-C infections and determine the origin, distribution, and diversity of aMPV-C viruses in chickens.

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Human Alveolar Echinococcosis in Kyrgyzstan

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Human echinococcosis is a reportable disease in Kyrgyzstan. Between 1995 and 2011, human alveolar echinococcosis increased from ≤3 cases per year to >60 cases per year. The origins of this epidemic, which started in 2004, may be linked to the socioeconomic changes that followed the dissolution of the former Soviet Union.

Alveolar echinococcosis (AE) is a devastating disease in humans, caused by the larval stage of the fox tapeworm, *Echinococcus multilocularis* (*1*). In the absence of treatment, the condition is often fatal, although expensive and successful treatment options are available and have recently been documented to be effective (*2*). The closely related parasite *E. granulosus,* commonly transmitted between dogs and livestock, causes cystic echinococcosis (CE) when it infects humans. CE has emerged throughout central Asia following the dissolution of the former Soviet Union and has been attributed to changes in animal husbandry practices, decline in veterinary public health services, and increases in dog populations (*3*). We report evidence of an emerging epidemic of human AE in the former Soviet Republic of Kyrgyzstan.

The Study

Echinococcosis is notifiable in Kyrgyzstan, a small, mountainous, central Asian country (199,900 km^2) of ≈5.5 million inhabitants. All confirmed diagnoses are reported to the Government Sanito-Epidemiology Unit in Bishkek. Cases are reported as either CE or AE by morphologic and histologic examination of resected lesions after surgical treatment. Age, sex, origin, and occupation of patients with reported cases are recorded. This reporting procedure has been used since the time of Soviet administration. However, the number of cases is likely underreported, and many case-patients with echinococcosis do not receive treatment because of widespread poverty and misdiagnoses. We analyzed the official reported cases of AE for the years 1995 through 2011 and present the annual numbers of all case-patients, categorized by sex and district of origin.

After the first AE case was recorded in 1996 in Kyrgyzstan, 0–3 cases occurred each year until 2003. Since 2004, the numbers of reported cases have increased substantially, reaching 61 in 2011 (Figure 1) ($p<0.0001$, χ^2 for trend). The total number of AE cases reported from 1995 through 2011 was 291. Of these, 185 case-patients were female and 106 case-patients were male ($p<0.0001$, χ^2). The mean age of case-patients was 33.4 years, with a range of 3–76 years. Eight cases occurred in children <10 years of age, and an additional 30 cases occurred in children 10–19 years of age (Table). The highest incidences were from the Osh, Issyk-Kul, and Naryn Oblasts (a subnational administrative division); the annual incidence in Naryn for the last 2 years of the study period was 7.1 cases per 100,000 population (Figure 2). All other districts had a mean annual incidence of <2 cases per 100,000, whereas the mean annual incidence nationwide is 1.2 cases per 100,000 with relatively few cases from the Batken and Talas Oblasts (Figure 2).

These data suggest an epidemic, at least as far as confirmed reported cases indicate, of AE in Kyrgyzstan that began in ≈2004. The case-patients reported here were also young (mean age 33 years) compared to case-patients in Europe, for example, in Switzerland, where the higher mean age is 54 years (*2*). Kyrgyzstan, however, has a young population; median age is 24.7 years, whereas the median age of those infected in Switzerland is 41.3 years (data from US Census Bureau, International Programs, www.census.gov/population/international/data/idb/informationGateway.php). This difference in the population pyramids means that Kyrgyzstan has a much higher proportion of children and young adults than Switzerland, and therefore a greater proportion of Kyrgyz case-patients would have been exposed and diagnosed at an earlier age.

Conclusions

Dogs are usually essential for CE transmission, and thus they are the key to the increasing human CE incidence reported previously (*3*). Dogs are highly susceptible to infection with *E. multilocularis* (*4*), and 18% of dogs from Naryn Oblast harbor this parasite, a similar prevalence to *E. granulosus* infection (*5*). Therefore, contact between humans and dogs responsible for transmission of CE could result in transmission of AE.

More than 90% of the estimated 18,000 annual new cases of AE throughout the world are in China, mainly the Tibetan plateau (*6*), where there is also a concomitant high prevalence of *E. multilocularis* infection in dogs (*7*). Dog contact has been reported as a risk factor for human AE on the Tibetan plateau (*8*). Therefore, *E. multilocularis* infection in dogs in Kyrgyzstan may be key in the

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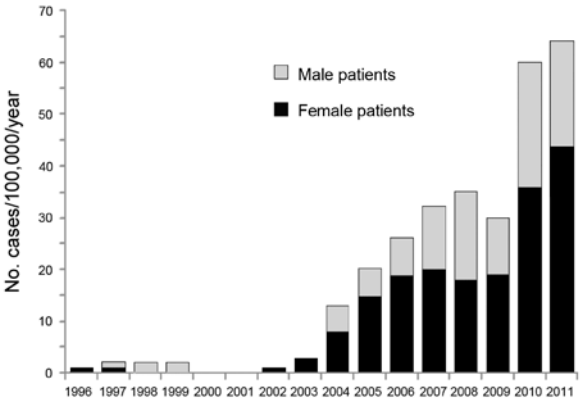

Figure 1. Number of alveoloar echinococcosis cases reported in Kyrgyzstan, by patient sex, 1995–2011.

Table. Distribution of alveolar echinococcosis cases by patient age and sex, Kyrgyzstan, 1995–2011

Age, y	No. patients		Total
	Male	Female	
<10	5	3	8
10–19	13	17	30
20–29	38	59	97
30–39	23	50	73
40–49	16	25	41
50–59	8	21	29
≥60	3	10	13

development of the reported epidemic of human AE. The parasite has colonized dogs because dog populations have increased and because dogs scavenge for rodents. Dogs that spend a large proportion of time untied, and thus are able to roam, have a higher prevalence of *E. multilocularis* infection in Kyrgyzstan (*5*).

This epidemic also appeared 10–15 years after the dissolution of the Soviet Union. This time frame coincides with the hypothesized latent period of human AE. In Switzerland, human AE has also emerged, but this has been linked to an increase in the fox population (*9*). Unlike the situation in Switzerland, we have no data on fox populations and thus cannot speculate about any possible changes in fox and small animal ecology that could be an alternative hypothesis for changes in AE incidence. However, the recent incidence in Kyrgyzstan is 4–5 times higher than it is in Switzerland, and therefore the epidemiology of transmission to humans is likely to be different. However, among foxes in Kyrgyzstan, prevalence of infection with *E. multilocularis* is 65%, with a mean abundance of >8,500 parasites per fox (*10*), and this abundance of parasites may be critical in transmission to humans if substantial fox–human contact occurs. Nevertheless, *E. multilocularis* infection in foxes in Kyrgyzstan is unlikely to be a recent phenomenon because *E. multilocularis* was reported in rodent intermediate hosts in the 1950s (*11*).

Lithuania, another former Soviet republic, also reported increased numbers of AE cases during 1997–2006 (*12*). In Lithuania, involvement of dogs in the transmission of *E. multilocularis* has been suggested. There is also a report of human AE emergence in Poland (*13*), another former communist country, with a steady increase in cases from 1990 to 2011. In Poland, the fox population is increasing, and many AE cases originate in areas with large fox populations. The role of dogs in transmission in Poland cannot yet be clarified because of lack of data.

This report does not prove that human AE has increased in Kyrgystan. It is possible that reporting of cases may have improved. Nonetheless, AE has long been known in the states of the former Soviet Union; cases were diagnosed in Russia as early as 1900 (*14*). Thus it seems unlikely that a failure to diagnose large numbers of AE cases would have occurred, which would have to have been the situation if the incidence of human AE had remained constant. Furthermore, medical services were adversely affected during a period of severe economic hardship after the dissolution of the Soviet Union, thus making improved diagnosis an unlikely factor in the increased number of cases reported. In the disease-endemic area in central Europe, the numbers of reported cases of AE have increased since the disease was first

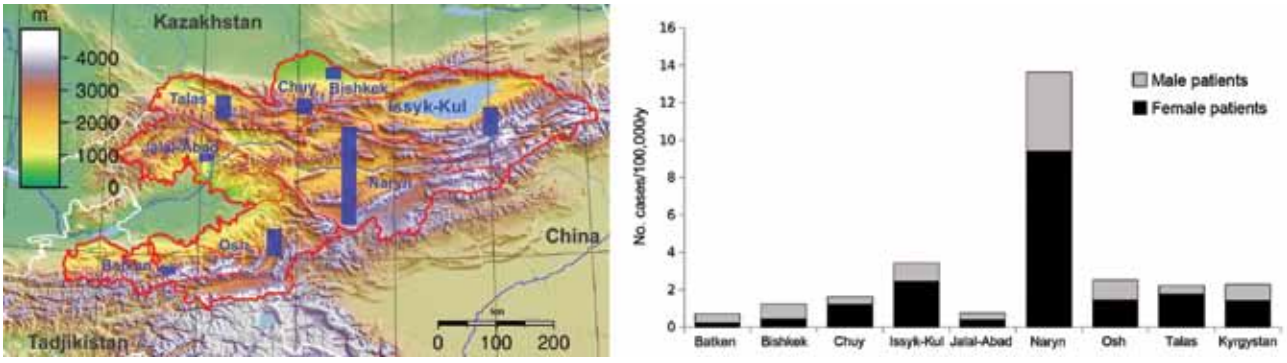

Figure 2. Relative incidence of alveoloar echinococcosis in Kyrgyzstan, by district, 1995–2011. The size of the bars on the map is proportional to the incidence.

described in the mid 19th century, and this increase may be due to improved diagnosis in recent decades (*14*). Increases in fox populations, particularly urban fox populations, may have provided the environment for increased transmission to humans (*15*), but to date, the only convincing evidence that this increase in fox populations has resulted in increased numbers of AE cases has come from Switzerland (*9*). In conclusion, AE appears to be a rapidly emerging problem in Kyrgyzstan, which may be related to profound socioeconomic changes that have occurred in the past 25 years.

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Molecular Epidemiologic Source Tracking of Orally Transmitted Chagas Disease, Venezuela

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Oral outbreaks of Chagas disease are increasingly reported in Latin America. The transitory presence of *Trypanosoma cruzi* parasites within contaminated foods, and the rapid consumption of those foods, precludes precise identification of outbreak origin. We report source attribution for 2 peri-urban oral outbreaks of Chagas disease in Venezuela via high resolution microsatellite typing.

Rapid urbanization presents new challenges for Chagas disease control in Latin America. Foci of disease are now reported in slums surrounding several Andean cities (*1*–*3*). Oral transmission is believed responsible for recent outbreaks of Chagas disease, most of which were characterized by atypically severe symptoms (*4*,*5*). Many cases have occurred in urban settings (*5*,*6*), amplifying the size and effect of the outbreaks.

Sources of orally transmitted disease outbreaks vary, but contaminated food and juices are often blamed. However, after a contaminated food is eaten, it may take weeks for the onset of clinical signs and symptoms, and direct molecular and cytological incrimination of a particular batch of food/beverage has not been possible (*5*). Thus, evidence pointing to particular foodstuffs is often circumstantial.

Molecular epidemiologic analyses of human and environmental isolates are routinely used to track the source of outbreaks caused by foodborne pathogens. High-resolution molecular markers have been developed and validated for *Trypanosoma cruzi*, the parasite that causes Chagas disease (*7*,*8*). These markers, used in conjunction with careful sampling, can identify the source of foodborne outbreaks.

The Study

We studied 2 outbreaks of orally transmitted Chagas disease (120 cases, 5 deaths). The first occurred in Chichiriviche, Vargas State, a coastal community (population ≈800 persons) ≈50 km northwest of Caracas, Venezuela. The outbreak occurred at a primary school where food was prepared on site. In early April 2009, a total of 71 children (6–13 years of age) who attended the morning school shift and 14 adults became ill. Exposure of these persons to *T. cruzi* was established by use of IgM and IgG ELISA. Parasitemia was observed in 33 of the patients with serologic results positive for *T. cruzi* infection (*9*,*10*).

The second outbreak occurred in Antimano, a peri-urban slum southwest of central Caracas (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/7/12-1576-Techapp1.pdf). In May 2010, 35 patients with suspected *T. cruzi* infection were examined at Hospital Miguel Perez Carreno in Caracas. Patients reported that they regularly ate at the same local communal canteen. Among the patients tested, 15 were positive for *T. cruzi* IgM and IgG (*9*). Parasitemia in 14 patients was confirmed indirectly by hemoculture. Of the 35 patients, 21 (2 adults, 19 children) were hospitalized.

To enable outbreak source attribution, we undertook intensive additional sampling of contemporary, nonhuman sources local to each outbreak and of human and nonhuman sources from more distant localities throughout Venezuela. In total, 246 *T. cruzi* strains and clones were typed for 23 microsatellite markers (online Technical Appendix 1 Table) (*8*). A list of the samples and their sites of origin is in online Technical Appendix 2 (wwwnc.cdc.gov/EID/article/19/7/12-1576-Techapp1.xlsx).

Individual level sample clustering was defined first by constructing a neighbor joining tree based on pairwise distances between multilocus genotypes (Figure 1). A second analysis used *K*-means clustering and discriminant analysis of principal components (Figure 2) (*11*). To assess connectivity between human and nonhuman outbreak cases, pairwise genetic differentiation (F_{ST}) was calculated (Table 1). Population-level genetic diversity was assessed first by calculating allelic richness then private allele frequency

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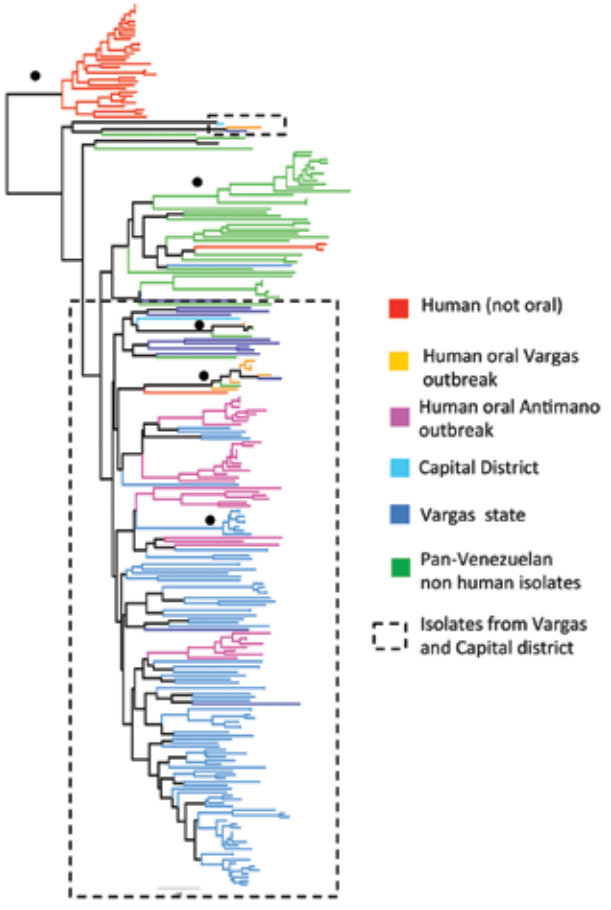

Figure 1. Unrooted neighbor joining tree showing genetic clustering among *Trypanosoma cruzi* isolates from 2 outbreaks of oral disease in northern Venezuela. Based on pairwise genetic distances (1 - proportion of shared alleles) between multilocus microsatellite profiles (23 loci) generated from 246 isolates and clones. Black circles indicate nodes with >60% bootstrap support. Branch color key is shown. Dashed boxes indicate isolates associated with the outbreaks.

over loci between each human–nonhuman population pair (Table 2). Geographic sampling distribution is shown in online Technical Appendix 1 Figure 2.

Clustering results determined by discriminant analysis of principal components and neighbor joining were broadly congruent. In the former, 19 clusters were defined; sample allocations are included in online Technical Appendix 2. Substantial overlap existed between some clusters, especially those from Capital and Vargas States (e.g., those labeled "x" in Figure 2), while others were highly distinct (e.g., cluster 8 in Figure 2). Human isolates from both oral outbreaks are extremely distinct from non-orally transmitted isolates collected from humans throughout Venezuela. Almost all these presumably vector-transmitted strains are closely related to one another, despite their geographic dispersal (cluster 8 in Figure 2). By comparison, oral outbreak strains that were isolated a mere 50 km apart (clusters 2, 5, 7, 9, 15–17) are far more globally diverse. Unlike most human isolates in Venezuela, which are distinct from nonhuman strains, oral outbreak isolates are interspersed among nonhuman strains from Venezuela. Furthermore, samples from both outbreaks clustered among nonhuman strains local to that outbreak, clearly indicating a local origin. Oral samples from each outbreak are polyphyletic with respect to strains from their immediate environment, a finding consistent with multiple contamination events or multiclonal infection sources.

F_{ST} values further support connectivity between outbreak and local environmental samples in both Antimano and Chichiriviche (Table 1). A lack of private alleles between human and nonhuman isolates also supports a local source for the Chichiriviche outbreak (Table 2). F_{ST} values in the 4-way comparison between outbreak and local environmental strains are, however, somewhat equivocal with respect to the entire dataset (Table 1). Cluster analysis showed that the human and nonhuman strains from Chichiriviche interspersed with strains from other states in Venezuela (Figure 2). Thus, we cannot confirm a uniquely local origin for the Chichiriviche outbreak, despite a low value for F_{ST}, and it is possible that some contaminating strains originated elsewhere.

Conclusions

This study demonstrates the value of rigorous molecular epidemiologic analysis of orally transmitted *T. cruzi* outbreaks, including the importance of appropriate sampling to identify the origin of the infecting strains. The foodstuff that propagated the peri-urban outbreak in Antimano was certainly contaminated locally. An active nonhu-

Table 1. F_{ST} values in a 4-way comparison for differentiation between *Trypanosoma cruzi* isolates derived from humans and the local environment during an outbreak of orally transmitted Chagas disease in 2 areas of Venezuela*

	Human isolates from		Nonhuman isolates from	
Isolate, location	Antimano	Chichiriviche	Antimano	Chichiriviche
Human				
Antimano		0.000	0.000	0.000
Chichiriviche	0.201		0.000	0.004
Nonhuman				
Antimano	0.093	0.170		0.000
Chichiriviche	0.088	0.053	0.079	

*Lower left shows linearized F_{ST} (genetic differentiation) values; upper right shows associated p values.

Table 2. Sample size corrected diversity between *Trypanosoma cruzi* isolates derived from humans and the local environment during an outbreak of orally transmitted Chagas disease in 2 areas of Venezuela*

Isolate, location	No. isolates/no. genotypes	Sample size corrected allelic richness ± SE	Mean no. private alleles/locus ± SE
Human			
Antimano	30/26	2.735 ± 0.291	0.32 ± 0.113
Chichiriviche	12/9	3.459 ± 0.412	0
Nonhuman			
Antimano	107/91	2.946 ± 0.320	0.86 ± 0.203
Chichiriviche	13/13	3.443 ± 0.409	0

man transmission cycle in the slums of Caracas, maintained by *Rattus rattus* rodents and *Panstrongylus geniculatus* triatomines, is the likely source. The Chichiriviche outbreak, however, has potential sources both in and outside the immediate area. As found in Chagas disease outbreaks linked to açaí palm fruit in Brazil (*12*), the *T. cruzi* parasite can survive for several days in some foodstuffs (*13*). Also, triatomines can survive for months in harvested crops; thus, multiple hygiene interventions are potentially necessary along the food production line (*14*). Nonetheless, if the foodstuff implicated was prepared locally, local contamination represents the most likely source of the outbreak. Study of additional nonhuman strains from Chichiriviche is necessary to support this assertion.

Crucial to understanding parasite transmission in general, we believe, are genetic differences between strains from orally and non-orally transmitted human cases. All TcI strains appear to be infective to humans and adapted to long-term carriage (*8*). However, the presence of a common, reduced-diversity TcI genotype cluster (TcI_{DOM}) among a high proportion of human Chagas disease cases in South America is also well established (*7*,*8*). We originally hypothesized that TcI_{DOM} was maintained, despite the presence of sympatric and infective sylvatic strains, because of low parasite transmission efficiency by invasive sylvatic vectors (*8*). Oral transmission is likely to be much more efficient. Thus, unlike TcI_{DOM} strains, those from orally transmitted *T. cruzi* cases demonstrated high genetic diversity and clearly originated from local nonhuman *T. cruzi* populations. However, it is also true that all TcI_{DOM} strains we isolated originated from patients with chronic infection, and all orally transmitted cases were in the acute phase. We cannot, therefore, rule

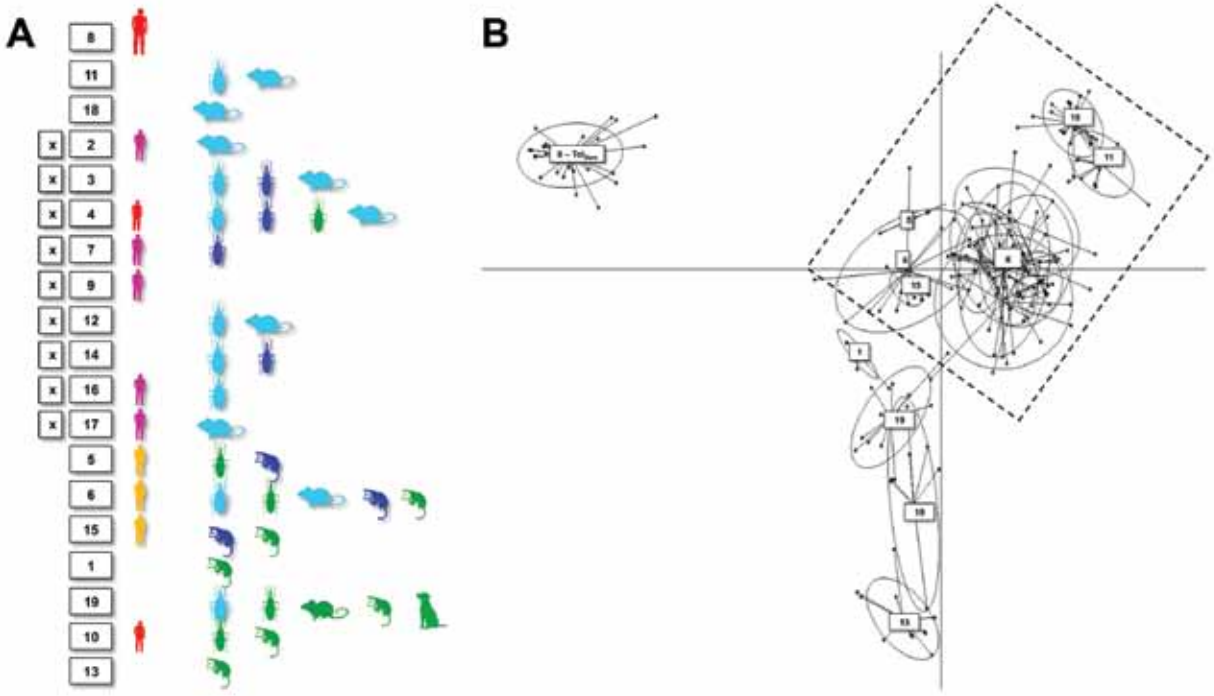

Figure 2. Discriminant analysis of principal components showing genetic clustering among *Trypanosoma cruzi* isolates from 2 outbreaks of oral disease in northern Venezuela. Six principal components were retained, explaining 80% of the diversity. Ellipses correspond to the optimal (as defined by the Bayesian information criterion minimum) number of population clusters among the genotypes analyzed. Images indicate sample host origin (human, rodent, marsupial, or triatomine), while colors correspond to the key in Figure 1. A full list of samples and population assignments (numbered boxes) is included in online Technical Appendix 2 (wwwnc.cdc.gov/EID/article/19/7/12-1576-Techapp1.xlsx). Dashed box indicates the isolates associated with the outbreaks.

out a role for immune selection in driving the frequency of TcI_{DOM} infections among humans; such selection represents an intriguing topic for future enquiry.

Molecular tools and reference datasets are now available to determine the source of acute Chagas disease outbreaks within days of their occurrence. The plummeting cost of such analyses means it is time to apply population genetic techniques and markers developed for trypanosomes as genuine epidemiologic tools.

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Unique Clone of *Coxiella burnetii* Causing Severe Q Fever, French Guiana

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Acute Q fever is an emergent and severe disease in French Guiana. We obtained 5 *Coxiella burnetii* isolates from samples of patients from Cayenne and found an epidemic clone circulating in Cayenne. This clone has caused pneumonia and endocarditis and seems to be more virulent than previously described strains.

Q fever, which is caused by the bacterium *Coxiella burnetii*, has rapidly emerged in French Guiana since 1996 (*1*). The incidence of acute Q fever in the capital, Cayenne, is one of the highest in the world. The annual incidence of Q fever was estimated at 37 cases/100,000 persons in 1996 (*2*) and increased to 150 cases/100,000 persons in 2005 (*3*). The most common clinical feature of Q fever in Cayenne is pneumonia, and *C. burnetii* is the causative agent of 24% of all community-acquired pneumonias (*4*). These forms of acute Q fever are particularly severe (*4*). Subsequently, we have hypothesized the existence of a specific source of *C. burnetii* responsible for human infections, which is unidentified to date, and the existence of a different strain of *C. burnetii* that circulates in Cayenne.

Q fever is diagnosed by serologic analysis in Cayenne. *C. burnetii* is rarely identified by PCR and has yet to be cultured in Cayenne. In this study, we isolated 5 *C. burnetii* strains from biologic samples of patients from Cayenne. We compared the strains from Cayenne with other strains and showed that a unique genotype is circulating in Cayenne. This unique genotype might be related to the clinical and epidemiologic features of severe fever in Cayenne.

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The Study

As a national reference center for Q fever, our center receives samples from France and other regions for serologic, molecular, histologic, and immunohistochemical analyses as described (*5*–*7*). In 2012, we received a cardiac valve sample from a patient in Cayenne with Q fever endocarditis who had undergone surgery in Martinique. In the same year, we collected 4 heparinized blood samples from patients with acute Q fever from Cayenne that were collected before initiation of antimicrobial drug treatment.

The patient in Cayenne who had endocarditis was a 60-year-old man. Serologic titers of IgG1 increased to 51,200 in this patient, and results of quantitative PCR (qPCR) and immunohistochemical analyses of the valvular sample were positive for *C. burnetii* (Figure 1). The other 4 patients (2 men and 2 women) had fever and acute pneumonia, and 2 of them also had increased transaminase levels. All 4 patients had serologic titers compatible with acute Q fever. The heparinized blood samples were tested by using a specific qPCR; samples from only 1 patient were positive for *C. burnetii*.

All samples were cultured as described (*8*). We successfully cultured *C. burnetii* from the valvular sample after incubation for 16 days and from 3 blood samples after incubation for 25, 32, and 32 days. Paradoxically, the only blood sample that was positive by qPCR was negative by culture. Genotyping was performed by using multispacer sequence typing for intergenic regions (*9*). We identified the isolate from the cardiac valve as genotype 17. Spacers Cox 51 and Cox 20 were the most discriminating spacers in identifying this genotype, and the 3 isolates from the blood cultures were also identified by these 2 spacers as genotype 17 (Figure 2). This genotype had been identified in our laboratory only once, in 2000 in an aortic valve of a 40-year-old French man who had undergone surgery in France, had Q fever endocarditis, and was co-infected with *Streptococcus oralis*. Retrospectively, we found that patient had lived in Cayenne for years before his diagnosis with Q fever endocarditis. Therefore, all genotype 17 isolates were obtained from who lived or had lived in Cayenne, making it unique to this area. We determined the antimicrobial drug susceptibilities of these isolates, and the MIC of doxycycline was 0.25 μg/mL for all isolates (*10*).

For the past 10 years, routine cell culture for *C. burnetii* has been performed in our laboratory. We found that the proportion of isolates obtained from blood samples of patients with acute Q fever was higher for patients from Cayenne than for patients from metropolitan France. We obtained 3 isolates from 5 blood samples from untreated patients in Cayenne and 3 isolates from 65 samples from patients in metropolitan France ($p = 0.003$, by Fisher exact

[1]These authors contributed equally to this article.

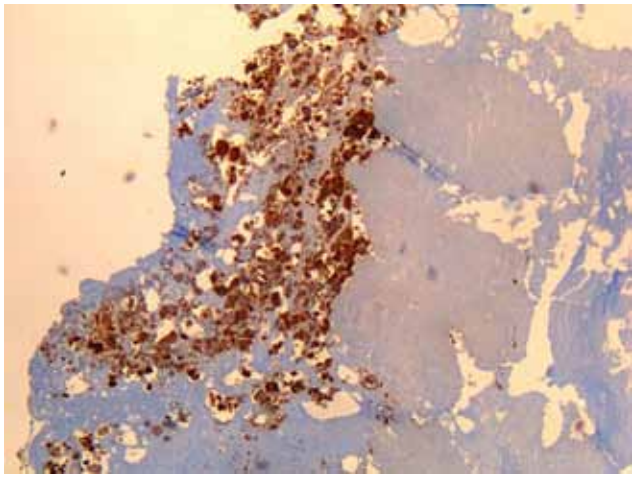

Figure 1. Immunohistochemical detection of *Coxiella burnetii* in resected cardiac valve of a 60-year-old man with Q fever endocarditis, Cayenne, French Guiana. Monoclonal antibody against *C. burnetii* and hematoxylin were used for staining. Original magnification ×50.

test). However, we did not find any difference with respect to the culture delay between patients from the 2 locations.

Conclusions

Our work shows that genotype 17, a unique genotype, is circulating in Cayenne. This genotype is related to genotypes that harbor the QpH1 plasmid, which causes the most severe clinical forms of acute Q fever in experimental animal models (*11,12*). Only bacteria from this group and bacteria containing the QpDV plasmid have been found in cases of human acute Q fever (*9,13*). We believe that severity of Q fever infection is related to the strain of *C. burnetii* circulating in Cayenne. Because the population in French Guiana is a large diversity of Creole, Amerindian, Maroon, Caucasian, and Asian persons (*14*), we excluded the hypothesis that genetic susceptibility of patients from Cayenne to *C. burnetii* infection might be related to severity of the disease.

We observed a higher rate of strain isolation from the blood of patients from Cayenne than from the blood of patients from metropolitan France. This difference may be related to a higher bacterial load in blood or a greater ability of the bacteria to grow on cell cultures. This second hypothesis seems more likely because even if the observed delay for the first culture was the same for genotype 17 and other genotypes, the strain from Cayenne was isolated more frequently in the cell line that we used (HEL cells) than other strains of *C. burnetii* from France. In addition, we did not find a higher number of DNA copies in the blood of patients from Cayenne, and qPCR results were not correlated with culture results.

C. burnetii isolates from Cayenne were susceptible to antimicrobial drugs, particularly doxycycline. These isolates cause acute pneumonia and endocarditis. Among the 34 genotypes identified in our study by multispacer

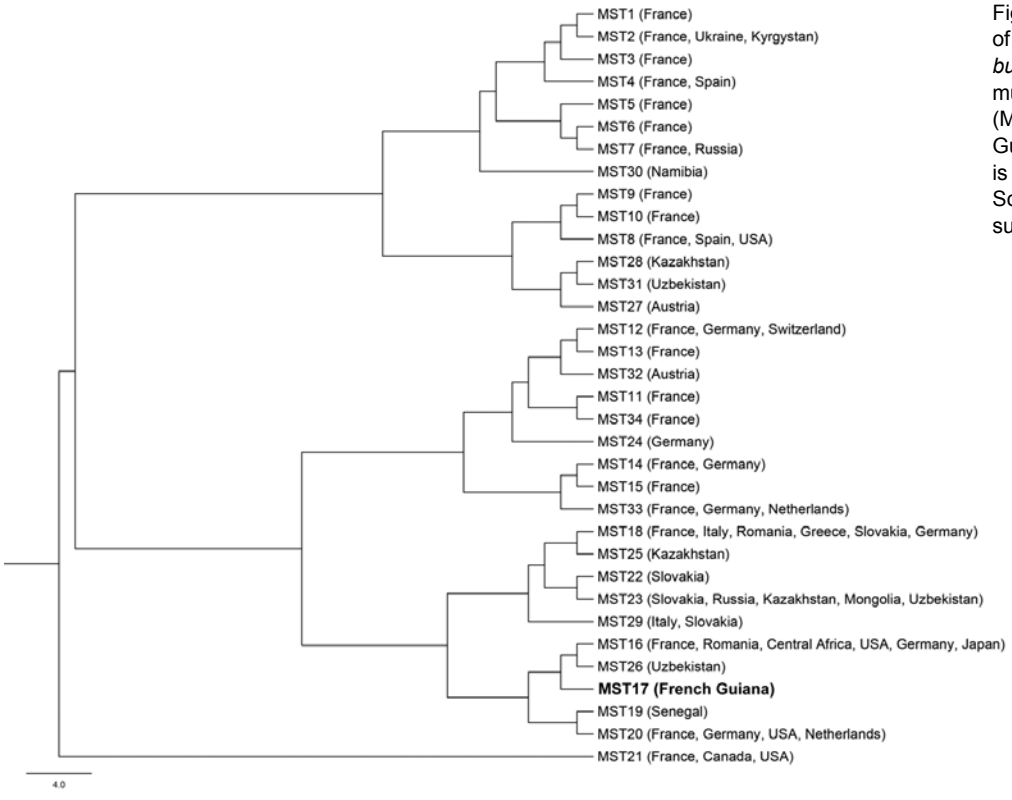

Figure 2. Phylogenetic diversity of 34 genotypes of *Coxiella burnetii* identified by using multispacer sequence typing (MST). Clone from French Guiana isolated in this study is indicated in **boldface**. Scale bar indicates nucleotide substitutions per site.

sequence typing, 28 (82%) were associated with disease in humans. A larger biodiversity of strains has been observed in samples from patients in metropolitan France (*9*), where 21 genotypes circulate (Figure 2). In contrast, during the recent Q fever outbreak in the Netherlands, it appears that a single strain (genotype 33) was responsible for the epidemic (*15*). We believe that there has been an epidemic developing in Cayenne since 1996 that is caused primarily, if not solely, by a single strain that has circulated since at least 2000, whose reservoir is unknown (*1,2*).

In conclusion, *C. burnetii* genotype 17 is circulating in French Guiana and causing acute infections and endocarditis. This strain is epidemic and most likely causes more acute infections with exacerbated immune responses than other known genotypes of *C. burnetii*. Genotype 17 might be the most virulent genotype of *C. burnetii* described to date.

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Dr Mahamat is an infectious diseases physician and head of the infection control unit at the General Hospital of Cayenne, French Guiana. His research interests include emerging disease surveillance, outbreak reports and quasi-experimental studies using times series analysis.

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Babesia microti Infection, Eastern Pennsylvania, USA

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Infection with *Babesia microti* has not been well-described in eastern Pennsylvania, USA, despite the vector of this organism being prevalent. We report 3 cases of babesiosis in eastern Pennsylvania in persons without recent travel outside the region or history of blood transfusions, suggesting emergence of this infection.

Babesiosis is an intraerythrocytic infection caused by protozoan parasites of the genus *Babesia*. In the United States, *Babesia microti* is the most common species that causes human babesiosis. Disease-endemic areas include specific regions in the northeast and upper Midwest United States. Infection with this organism can be asymptomatic to life-threatening. Signs and symptoms include high fever, diaphoresis, chills, headaches, and anorexia. Patients can also have hemolytic anemia and thrombocytopenia (*1*).

B. microti is transmitted by the *Ixodes scapularis* tick, which is also the vector of *Borrelia burgdorferi* and *Anaplasma phagocytophilum* (*2*). Although *B. burgdorferi* is endemic to Pennsylvania (*3*), *B. microti* is not considered endemic to this region (*4,5*). We report 3 cases of human babesiosis in patients from Northampton County in eastern Pennsylvania, USA, who had not recently traveled outside the region or had blood transfusions. None of the patients had risk factors for severe babesiosis, such as asplenia.

The Patients

Patient 1 was a 68-year-old man who was hospitalized on August 18, 2011, because of 6 days of fever, arthralgias, generalized weakness, and confusion. He was given doxycycline for treatment of presumptive Lyme disease but showed no improvement. He had not traveled outside eastern Pennsylvania for >3 years. He had never received any blood transfusions. Although he did not recall any tick attachments, he enjoyed gardening and other outdoor activities.

Pertinent clinical and laboratory data are shown in the Table. During testing for thrombocytopenia, a peripheral blood smear was obtained on hospital day 1 and showed ring forms with tetrads (Figure) and a parasitemia level of 10%. A PCR result for the blood sample was positive for *B. microti*.

Despite treatment for 5 days with clindamycin and quinine, the treatment of choice for severe babesiosis, the fever persisted. Antimicrobial drug therapy was changed to atovaquone and azithromycin. The patient completed 10 days of treatment and showed resolution of symptoms and normalization of platelet count and total bilirubin level. Parasitemia level at the time of discharge was 1.2%. Laboratory data after discharge were not available.

Patient 2 was an 84-year-old woman with microcytic anemia who was hospitalized on June 8, 2012, because of 2 weeks of fever, diaphoresis, myalgias, progressive dyspnea, and fatigue. Originally from New Hampshire, she had been living in Northampton County, Pennsylvania, for >4 years. She had not traveled outside the region in the past 4 years. She received a blood transfusion for chronic anemia 1 year before onset of this illness. She recalled multiple tick bites in the recent past.

Because of pancytopenia (Table), a bone marrow biopsy was performed and showed intraerythrocytic ring forms with tetrads. Parasitemia level for a peripheral blood smear was 1.4%. A PCR result for *B. microti* was positive.

The patient was given atovaquone and azithromycin for 10 days and showed resolution of symptoms and improvement in abnormal laboratory values. A repeat blood smear on June 18 showed a parasitemia level of 0.1%, and a blood smear 1 month later showed no parasites.

Patient 3 was a 71-year-old man who was hospitalized on June 26, 2012, because of 2 weeks of fever and gradually worsening malaise and weakness. He had not traveled outside eastern Pennsylvania in the past 2 years and never received a blood transfusion. Two weeks before admission, he had an insect bite that developed into a larger, oval rash.

Initial testing for thrombocytopenia included a peripheral blood smear, which showed intraerythrocytic ring forms and tetrads and a parasitemia level of 0.4%. A PCR result was positive for *B. microti*. He was given atovaquone and azithromycin for 10 days. Because results of enzyme immunoassay and Western blot for *B. burgdorferi* were positive, he was also given doxycycline. Symptoms resolved, and the laboratory values improved. A repeat blood smear 1 week after starting antimicrobial drug therapy showed no parasites.

Conclusions

This report supports the hypothesis that babesiosis caused by *B. microti* is emerging in eastern Pennsylvania. As *B. microti* is spread by *I. scapularis* ticks, this infection might emerge in the range of the vector. Babesiosis is considered endemic to some states in which *I. scapularis* ticks are prevalent (*6,7*), but not in Pennsylvania.

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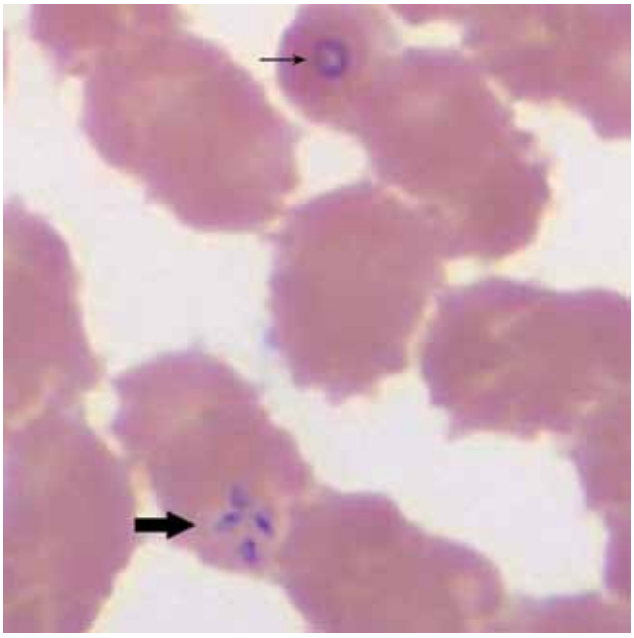

Figure. Wright-stained blood smear for patient 1 with babeosis on day 1 of hospitalization, eastern Pennsylvania, USA, showing an intraerythrocytic trophozoite of *Babesia microti* in a ring form (thin arrow) and a tetrad arranged in a cross-like pattern (thick arrow). Original magnification ×1,000.

All of these cases were confirmed by using robust laboratory methods. The 3 cases showed intraerythrocytic tetrad forms, an uncommon finding that is considered pathognomonic for *Babesia* infection (*1*). The strength of the diagnoses was further enhanced by PCR testing. Although PCR will amplify all *Babesia* species that are pathogenic in humans, the peak melting point temperature of amplicons for the 3 samples was nearly identical (difference ≤0.2°C) to that of the control *B. microti* amplicon, supporting *B. microti* as the causative agent (C.P. Cartwright, ViroMed Laboratories, Minnetonka, MN, USA, pers. comm.).

Two other persons with babesiosis have been treated at our institution since 2011. These case-patients were not included in this report because of inadequate laboratory confirmation for 1 patient and lack of a strict travel limitation for the other patient. One of these patients was given a diagnosis of babesiosis on the basis of serologic data obtained 2 months after the initial illness without blood smear confirmation. The second patient had traveled to western New Jersey, a babesiosis-endemic region, over a brief period before his illness.

The Pennsylvania Department of Health has received 39 voluntary reports of smear-positive babesiosis during 2005–2012 (K. Waller, Pennsylvania Department of Health, Harrisburg, PA, USA, pers. comm.). More than 60% of those reports were made after babesiosis became nationally reportable in 2011. Although many of these cases lacked detailed historical information to confirm that the cases were acquired in Pennsylvania, they further support the notion that B. microti is emerging in Pennsylvania.

Data on the prevalence of *B. microti* in *I. scapularis* ticks in eastern Pennsylvania are limited. However, 0.7% (3/443) of ticks collected from animals and humans in Monroe County in eastern Pennsylvania during 2004–2006 were infected with B. microti (*3*), supporting the plausibility of zoonotic transmission in the state.

Before these patients were identified, no cases of babesiosis had been diagnosed at our tertiary care center over the previous 10 years. Cases could have been missed because of the nonspecific nature of symptoms. However, this increase in the number of documented cases in 1 hospital is unlikely to be entirely explained by missed diagnoses. Furthermore, no other cases of babesiosis have been described at other institutions in this region.

This study had a few limitations. Recall bias could have played a role in obtaining a travel history. However, for each of these patients, family members were able to confirm the travel history, making recall bias unlikely. One of the patients had a blood transfusion 1 year before onset of systemic symptoms, making a transfusion-related infection unlikely. However, because this same patient was originally from New Hampshire, she might have been a chronic carrier.

These cases support the premise that babesiosis caused by *B. microti* infection is emerging in eastern Pennsylvania. Knowledge of the geographic distribution of *B. microti* is essential. Diagnosis requires strong clinical suspicion and supportive laboratory data. Timely diagnosis and treatment

Table. Clinical and laboratory data for 3 patients with babesiosis at dates of admission, diagnosis of babesiosis, and discharge, eastern Pennsylvania, USA*

Characteristic	Patient 1		Patient 2			Patient 3	
Date	2011 Aug 18	2011 Aug 25	2012 Jun 8	2012 Jun 12	2012 Jun 16	2012 Jun 26	2012 Jul 11
Temperature, °C	38.7	35.7	36.8	37.2	36.1	37.8	37.2
Hematocrit, %	43.1	28.3	26.0	27.4	30.7	43.3	32.9
Leukocyte count, × 10^9/L	9.87	6.43	2.94	5.47	3.36	7.93	10.6
Platelet count, × 10^9/L	45	197	46	68	68	41	74
Total bilirubin, mg/dL†	4.77	0.88	3.05	NA	NA	2.0	2.48
Parasitemia level, %	10.0	1.2	NA	1.4	0.1	0.4	0.0
Creatinine, mg/dL‡	1.9	1.1	1.47	1.12	NA	1.16	0.95

*NA, not available.
†Reference range 0.20–1.00 mg/dL.
‡Reference range 0.60–1.30 mg/dL.

for patients and testing of blood donors in areas in which *B. microti* is found might further prevent transfusion-related infection (*8–10*). Having this infection reportable in Pennsylvania and other states to which *I. scapularis* ticks are endemic might help identify the geographic region of this parasite.

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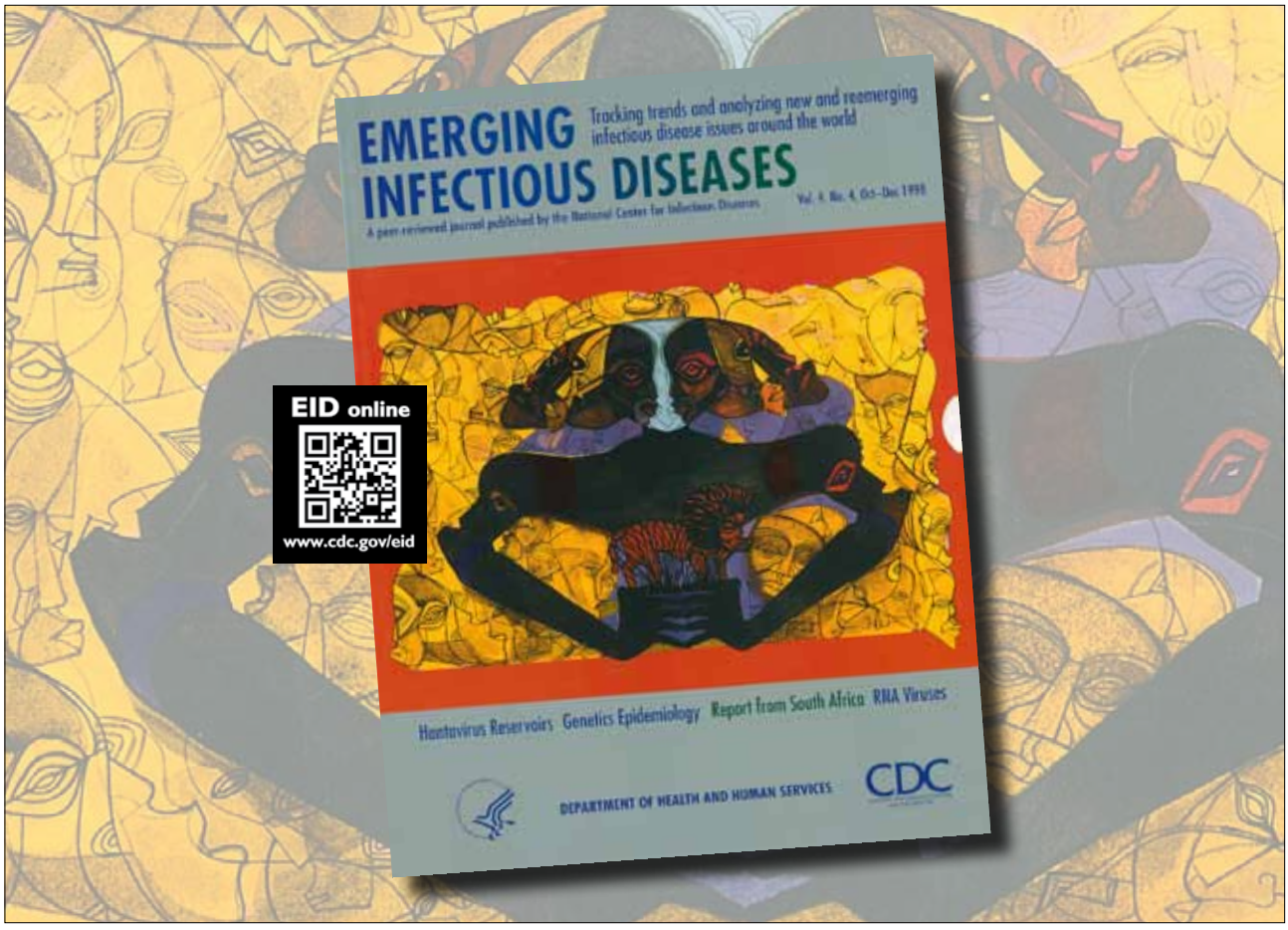

Reemergence of Chikungunya Virus in Bo, Sierra Leone

Rashid Ansumana, Kathryn H. Jacobsen, Tomasz A. Leski, Andrea L. Covington, Umaru Bangura, Mary H. Hodges, Baochuan Lin, Alfred S. Bockarie, Joseph M. Lamin, Moses J. Bockarie, and David A. Stenger

We diagnosed 400 possible IgM-positive cases of chikungunya virus in Bo, Sierra Leone, during July 2012–January 2013 by using lateral flow immunoassays. Cases detected likely represent only a small fraction of total cases. Further laboratory testing is required to confirm this outbreak and characterize the virus.

Outbreaks of infection with chikungunya virus (CHIKV), an alphavirus that is transmitted by bites of infected *Aedes* spp. mosquitoes, were frequent in sub-Saharan Africa and southern and Southeast Asia during the 1950s–1970s, but the infection largely disappeared in the 1980s; only sporadic cases were observed (*1*). The virus reemerged in the early 2000s; major outbreaks were reported in Kenya, some island nations in the Indian Ocean, and several countries in Asia (*2*,*3*).

The primary symptoms of CHIKV infection are high fever (>38.5°C [102°F]) and severe pain in the distal joints of the extremities or the lumbar spine. A maculopapular rash, sensorineural impairment, severe headache, and other nonspecific symptoms may also occur. Symptoms usually resolve within 1–2 weeks after onset of fever, but for a sizeable proportion of patients, arthralgia and arthritis become chronic and pain persists for years (*2*,*3*).

A nationwide serosurvey in Sierra Leone in 1972 detected cases of CHIKV infection throughout the country (*4*), but we are not aware of any cases reported since the mid-1970s. Two recent developments made reemergence appear imminent. First, outbreaks of reemerging CHIKV have been reported in neighboring Guinea (*5*) and in Senegal (*6*). Second, recent yellow fever cases in Sierra Leone have shown that *Aedes* spp. mosquito–borne infections are common (*7*). Thus, it was not surprising when we initiated an infectious disease surveillance study in July 2012 in the city of Bo, in Southern Province, Sierra Leone, that we detected possible chikungunya virus infections. We report initial results of our investigation.

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The Study

On July 7, 2012, the Mercy Hospital Research Laboratory (MHRL) in Bo, Sierra Leone, initiated a 1-year infectious disease surveillance program to identify the diversity of pathogens causing febrile illness in the city. A tiered analysis approach was used. First, all specimens from febrile study participants were tested for ≈12 infections with various pathogens, including CHIKV, by commercially available test kits. Specimens that showed negative results in this first round of testing were further tested by using cultures, multiplex PCR, and resequencing pathogen microarrays. The research protocol was approved by Njala University, George Mason University, the Liverpool School of Tropical Medicine, the US Naval Research Laboratory, and the Sierra Leone Ethics and Scientific Review Committee.

During July 7, 2012–January 10, 2013, MHRL conducted first-tier lateral flow immunoassay (LFI) tests of blood samples from all 932 outpatients ≥5 years of age who had been clinically examined at the hospital, were found to have febrile illness, and consented to having blood drawn for laboratory testing. LFI test kits (SD Bioline; Standard Diagnostics, Inc., Seoul, South Korea) were used for diagnosis of IgM against CHIKV; IgG and IgM against dengue virus and hepatitis A virus; hepatitis B virus surface antigen, hepatitis C virus, HIV-1/2, and antibodies against these viruses; and IgG and IgM against *Leptospira* spp., *Salmonella enterica* Serovar typhi, and syphilis.

Most patients reported that they had sought medical care within several days after the onset of their febrile illnesses. Levels of IgM against CHIKV are usually detectable by immunochromatographic methods within a few days after infection and persist for ≈3–4 months (*1*,*2*). The LFI test kits for CHIKV were reported by the manufacturer to have a sensitivity of 97.1% and a specificity of 91.1% compared with those of ELISA (*8*). An independent evaluation found a sensitivity of 50.8% and a specificity of 89.2% for the kits; sensitivity ranged from 40.9% 1–5 days after onset of illness to 65.4% 16–20 days after onset (*9*). Specificity decreases after the first week (*10*).

More than half of the cases tested during the first week of the surveillance program were positive by LFI for CHIKV. Thus, we notified the Sierra Leone Ministry of Health and Sanitation of a possible CHIKV outbreak. By January 10, 2013, 400 (42.9%) of 932 febrile patients were positive by LFI for CHIKV (Figure 1). Ages of the 400 CHIKV IgM-positive patients ranged from 6 years to

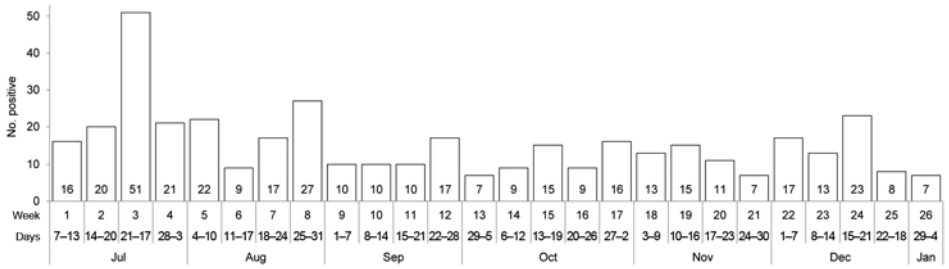

Figure 1. Weekly number of IgM-positive chikungunya virus test results at Mercy Hospital Research Laboratory, Bo, Sierra Leone, July 7, 2012–January 4, 2013.

85 years; 172 (43.0%) were male patients. Of these 400 patients, 220 (55.0%) reported arthralgia, 189 (47.3%) chills, and 156 (39.0%) headaches. Co-infections were common; 92 (23.0%.) were co-infected with malaria, 37 (9.3%) with HIV, 33 (8.3%) with hepatitis B virus, and smaller numbers with hepatitis A, hepatitis C, tuberculosis, typhoid, and syphilis. Four CHIKV-positive samples were also positive for dengue.

On July 28, MHRL launched an Ushahidi-based website (www.ushahidi.com) to compile case reports. Details about the patients who were positive for CHIKV were uploaded to the MHRL website (www.mhrlsl.com/GIA/ushahidi) and, if possible, were geolocated on an open street map (www.openstreetmap.org) that linked to a map of Bo created by MHRL for health research purposes (*11*). The map showed that the cases were located throughout Bo (Figure 2). Of the 400 LFI-positive case-patients, 319 (79.8%) could be mapped; the remainder did not provide a home street address on the laboratory patient information form. However, the sample was not population-based because Mercy Hospital is 1 of several hospitals serving Bo, so a city-wide attack rate could not be determined.

Results of attempts by the US Naval Research Laboratory to confirm the LFI results by using semi-nested reverse transcription PCR on fast technique for analysis of nucleic acid–preserved samples were inconclusive, possibly because of genetic sequence variation from well-characterized strains or because of the timing of specimen collections. Viral loads for humans with CHIKV infection decrease after the second day of symptoms, and viral titers may be low after the fifth day (*12,13*). Because CHIKV nucleic acids are only detectable in serum for a few days, reverse transcription PCR results are often discordant with those of serologic (IgM and IgG) assays. Confirmation that

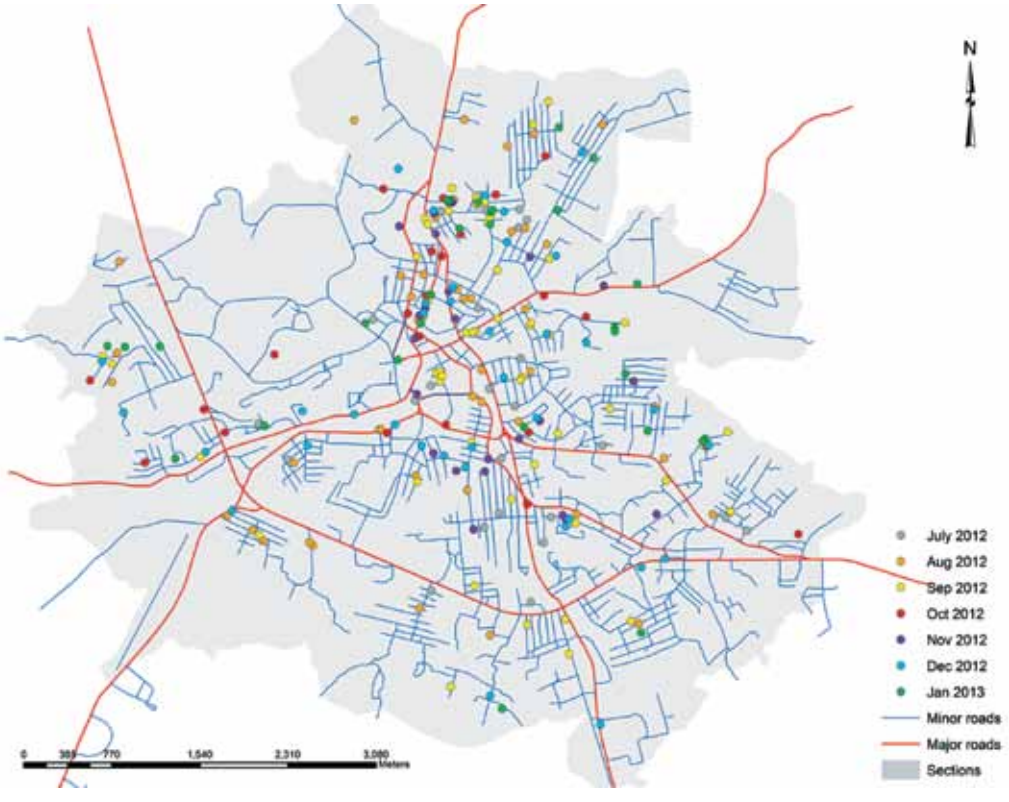

Figure 2. Residence locations for IgM-positive cases of infection with chikungunya virus, Bo, Sierra Leone, July 7, 2012–January 4, 2013.

an outbreak occurred is dependent on isolation of the virus, followed by molecular characterization, full-genome sequencing, and phylogenetic mapping.

Conclusions

Our results suggest that an outbreak of chikungunya virus occurred in Sierra Leone. The exact time of the reemergence of this virus cannot be pinpointed, but retrospective analyses of outpatient charts suggested that, on the basis of syndromic criteria, the first cases occurred in January 2012 and the outbreak peaked during the rainy season in 2012. Other outbreaks reported in central and west Africa have also occurred during the rainy season, which is typical for *Aedes* spp. mosquito–borne infections (*6*,*14*,*15*). Because Mercy Hospital serves only a relatively small proportion of the residents of Bo, the cases detected likely represent only a small fraction of the total cases that have occurred. Further study will be required to confirm the laboratory results and, if further investigation is warranted, to document the extent of the outbreak.

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Novel *Bartonella* Agent as Cause of Verruga Peruana

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While studying chronic verruga peruana infections in Peru from 2003, we isolated a novel *Bartonella* agent, which we propose be named *Candidatus* Bartonella ancashi. This case reveals the inherent weakness of relying solely on clinical syndromes for diagnosis and underscores the need for a new diagnostic paradigm in developing settings.

Bartonellosis is a disease caused by infection with species from the *Bartonella* genus. In South America, infection with *B. bacilliformis*, an α-2 proteobacterium, may cause a life- threatening bacterial infection (*1*,*2*). If untreated, the acute form of the illness, sometimes referred to as Oroya fever, has a high mortality rate because the bacteria invade erythrocytes, resulting in subsequent severe anemia and secondary infections. A chronic phase, termed verruga peruana, is characterized by vasculoproliferative skin lesions; some reseachers have also described an asymptomatic bacteremic phase, which may contribute to the longevity of the reservoir status of infected persons (*3*).

In 2007, a novel species of *Bartonella* (*B. rochalimae*) was isolated from a single traveler who had an acute febrile anemia after traveling to Peru (*4*). We report the identification of another novel agent of *Bartonella* isolated from a patient with chronic bartonellosis (collected in 2003, fully characterized in 2011–2012). We suggest that the isolate be named *Candidatus* Bartonella ancashi in honor of the highland region of Peru.

The Study

The patient was a 3-year-old boy with no known underlying medical history who was identified in 2003 as having clinical verruga peruana by the classic appearance of the eruptive nodular rash (Figure 1). He and his family lived in a rural setting near the town of Caraz, Ancash region, Peru. He lived in close proximity to numerous pets and farm animals and had experienced insect bites around the time the eruptive rash developed. His rash had been present for »30 days, and he had no fevers, chills, or arthralgias. Baseline laboratory studies included complete blood counts and bacterial culture for *Bartonella* species, using methods previously described (*5*,*6*). Briefly, the media was biphasic, consisting of Bacto agar with Proteose Peptone No. 3 (Becton, Dickinson and Co., Sparks, MD, USA), dextrose, sodium chloride and 10% defibrinated sheep's blood, and RPMI supplemented with 10% inactivated fetal bovine serum.

The physical examination revealed that the child had 56 lesions, distributed mainly on the extremities. Laboratory values were the following: hemoglobin level 12.8 mg/dL (reference range 11–13 g/dL), hematocrit 39% (reference range 31%–43%), and platelet count of 300,000/μL (reference range 15,000–400,000); his leukocyte count was elevated at 28,000/μL (reference range 4,100–10,900) with 51% eosinophils, for which he was referred for further evaluation.

The peripheral blood smear was negative for intracellular organisms, but blood culture was positive for a *Bartonella* species. This species was further studied and found to be novel on the basis of genetic sequencing of the isolate obtained from the standard blood culture (*7*). His condition was treated with azithromycin, and the rash fully resolved (*8*,*9*). To confirm the identity of the isolate from this patient, in 2011–2012 we conducted molecular analyses (including PCR, nested PCR [nPCR], and sequencing) on the whole blood culture isolate, *Bartonella* species no. 20.00 (*10*).The isolate was characterized by sequencing 3 gene fragments.

The following conditions were used for PCR amplification. For *rrs*, initial denaturation was at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and elongation at 68°C for 90 s for PCR and 70 s for nPCR, and then by a final extension step at 72°C for 7 min. For *gltA*–95°C, denaturation was for 1 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s, and elongation at 68°C for 60 s, and then by the final extension step at 72°C for 7 min. For *rpoB*, primers were selected from the conserved regions of RNA polymerase β-subunit encoding gene (*rpoB*) after alignment of the *rpoB* from *B. quintana* and *B. vinsonni* for PCR and nPCR. PCR and nPCR were carried out by using conditions identical to those described for *gltA* (Table).

PCR products were purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA) before sequencing. PCR products were sequenced in both directions by using the BigDye Terminator v3.1 Cycle Sequencing

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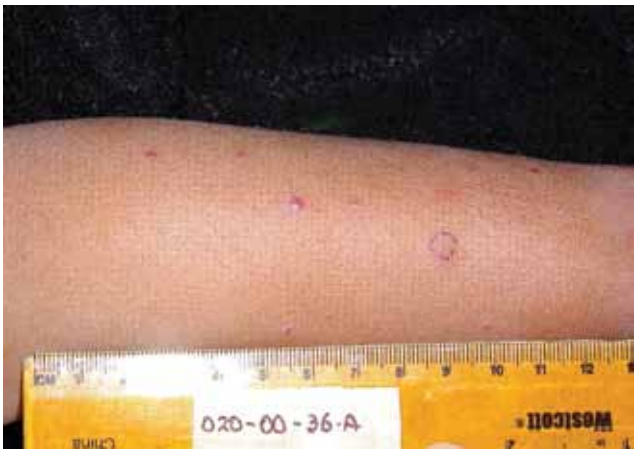

Figure 1. Clinical presentation of verruga peruana in 3-year-old boy, Peru, 2003.

Kit (Life Technologies, Carlsbad, CA, USA) and run on an automated 3130*xl* Gene Analyzer (Life Technologies). Sequences were characterized by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence analysis was performed by using BioEdit version 7.1.3 (Ibis Biosciences, Carlsbad, CA, USA). The multiple sequence alignments were performed with the ClustalW multiple alignment application also in BioEdit version 7.1.3. Phylogenetic trees were created with MEGA5 software using the neighbor-joining tree method with 1,000 bootstrap replicates (*11*).

Subsequent comparison with known *Bartonella* species in GenBank found no identical sequences. The 1,351-bp sequence of the *rrs* fragment was found to be 99.0% similar to the *rrs* fragment of *B. bacilliformis*. The 312- and 589-bp fragments of *gltA* and *rpoB*, respectively, were found to be most similar to their counterparts of *B. bovis* at 89.4% (*gltA*) and 85.9% (*rpoB*), respectively (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/12-1718-Techapp1.pdf). The sequence similarity ranges for the *rrs*, *gltA*, and *rpoB* for recognized *Bartonella* species are 97.7%–99.8%, 83.4%–96.1%, and 85.9%– 96%, respectively (*12*). In addition, *rpoB* and *gltA* are believed to have the best discriminating power for *Bartonella* species (*13*). La Scola et al. proposed that a new species be designated if the sequence similarities are <96% and <95.4% for a 327-bp fragment of *gltA* and a 825-bp fragment of *rpoB*, respectively (*13*). The sequence similarities for *Candidatus* Bartonella ancashi 20.00 to other known *Bartonella* species fall well below these suggested values, providing more evidence that this agent is unique. Phylogenetic analysis of the *rrs*, *gltA,* and *rpoB*, gene fragments provide additional evidence for identification of a unique *Bartonella* agent. The concatenated sequence of *gltA* and *rpoB* gene fragments placed the new *Bartonella* isolate in an exclusive clade that is most closely aligned with *B. bacilliformis* (Figure 2). The *rrs* fragment also placed the isolate in a clade with *B. bacilliformis*. The results from the phylogenetic analysis combined with the sequence similarity data provide evidence that this isolate, *Candidatus* Bartonella ancashi 20.00, is unique (*12*,*13*).

Conclusions

This case underscores the inherent weakness of relying solely on clinical syndromes for diagnosis. The variety of bacteria that have been implicated in the clinical spectrum of bartonellosis is increasing as molecular methods are applied to isolates that previously were identified by using clinical criteria or biochemical testing. The novel bacterium may have similar epidemiologic, clinical, and microbiologic properties to *B. bacilliformis*, but without relating these data to a full molecular characterization, that assumption is precarious.

To address this public health deficiency, a new diagnostic paradigm should be deployed to developing settings such as Peru. This is particularly true for areas with high biodiversity, a point identified by other investigators who have termed these regions "hot zones" for emerging infectious diseases (*14*). Unfortunately, tools such as high-throughput sequencing are rare in developing settings where risk for novel pathogen emergence is highest. Investment in advanced molecular diagnostic platforms in the developing setting will be an essential tool for expanding pathogen discovery; of course, this should be accompanied by parallel investments in training in molecular

Table. Primers used for PCR, nested PCR, and sequencing of novel *Bartonella* isolate from Peru, 2011–2012*

Gene	Primer name	Primer sequence, 5' → 3'	Use	Fragment length
rrs	16SU17F	AGAGTTTGATCCTGGCTCAG	PCR, nPCR, sequencing	1,424 bp
	16SU1592R	AGGAGGTRATCCAGCCGCA	PCR, nPCR, sequencing	
	16SU 833R	CTACCAGGGTATCTAATCCTGTT	nPCR, sequencing	
	16S E. coli-518F	CAGCAGCCGCGGTAATAC	nPCR, sequencing	
gltA†	BHCS 781p (F)	GGGACCAGCTCATGGTGG	PCR, sequencing	338 bp
	BHCS 1137n (R)	AATGCAAAAAGAACAGTAAACA	PCR, sequencing	
rpoB	BrpoB1435F	CGCATTGGTTTRCTTCGTATG	PCR	589 bp
	Brpo2327R	GTAGACTGATTAGAACGCTG	PCR, nPCR, sequencing	
	Brpo1696F	CCTACGCATTATGGTCGTATTTG	nPCR, sequencing	

*nPCR, nested PCR.
†*gltA* primers were previously described by Eremeeva et al. (*4*).

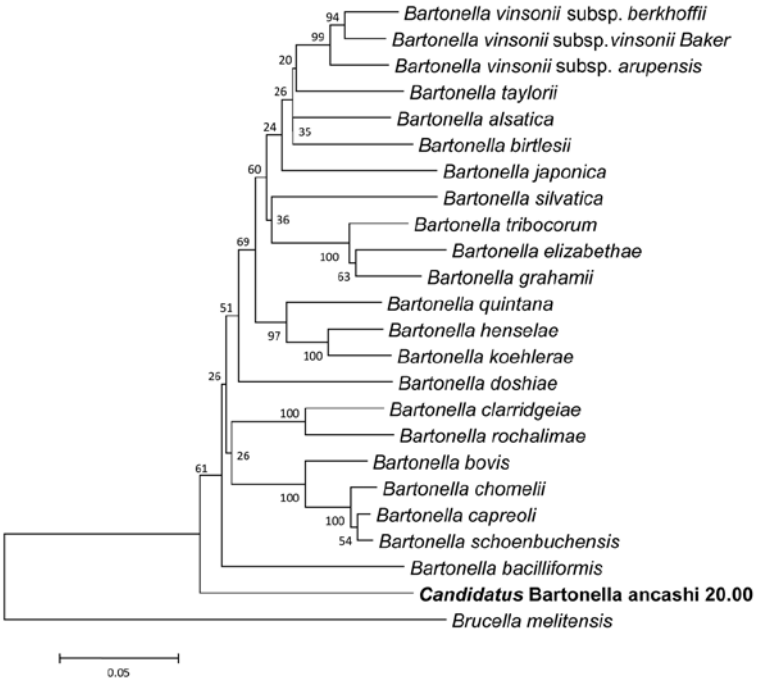

Figure 2. Phylogeny for concatenated sequences of novel *Bartonella* isolate (**boldface**), including a 312-character fragment of *gltA* and a 589-character fragment of *rpoB*. The neighbor-joining tree method (1,000 bootstrap replicates) was employed using MEGA5 software (*11*), and the distances were calculated by using the Jukes-Cantor method, in which units are calculated as the number of base pair substitutions per site (*10*). *Brucella melitensis* was used as the outgroup.

laboratory techniques and analysis for resident scientists. Opportunities for grants and stable faculty positions must also be supported to encourage qualified scientists to remain in the developing setting.

Finally, evidence indicates that humans contract bartonellosis only once and that lifelong immunity results from that primary infection (*15*). Because of this circumstance, and the inability to identify an animal reservoir of *B. bacilliformis*, Peruvian scientists and others have identified bartonellosis as a disease that may be eradicated in the Andean region through development of a vaccine against *B. bacilliformis*, targeted treatment of patients, and vector control programs (*15*). This possibility may be less feasible if multiple species of *Bartonella* cause bartonellosis. Further molecular and immunologic studies should be undertaken if this disease is to be targeted for eradication.

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We thank Jesus Gonzalez for his efforts in conducting the clinical trial in Peru.

The 3 genes have been submitted to GenBank, with the following accession numbers: *rrs*, KC178617, *gltA*, KC178618, *rpoB*. KC178619.

The original clinical trial was approved by the Institutional Review Boards of the Uniformed Services University of the Health Sciences, the Naval Medical Research Center, and the Universidad Peruana Cayetano Heredia in 2002, and compared the standard of care medication, rifampin, with azithromycin. The clinical trial was originally funded by Pfizer and the subsequent pathogen characterization was funded by the Department of Defense Global Emerging Infection System work unit no. 0000188M.0931.001.A0074. The funding agencies had no role in writing, editing, or approval of this manuscript.

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Schmallenberg Virus among Female Lambs, Belgium, 2012

François Claine, Damien Coupeau, Laetitia Wiggers, Benoît Muylkens, and Nathalie Kirschvink

Reemergence of Schmallenberg virus (SBV) occurred among lambs (n = 50) in a sheep flock in Belgium between mid-July and mid-October 2012. Bimonthly assessment by quantitative reverse transcription PCR and seroneutralization demonstrated that 100% of lambs were infected. Viremia duration may be longer in naturally infected than in experimentally infected animals.

During late summer and fall 2011, a nonspecific febrile syndrome characterized by hyperthermia, decreased milk production, and diarrhea occurred among lactating cows in Germany. A new virus, named Schmallenberg virus (SBV), was identified as the cause (*1*). This arbovirus of the genus *Orthobunyavirus*, family *Bunyaviridae*, affects domestic and wild ruminants and has been documented in Western European countries since 2011 (*2*). The most notable consequences of this new pathogen are caused by its ability to cross the placental barrier. Depending on the gestational age of the offspring, abortion, stillbirth, or severe congenital malformations, including arthrogryposis and defects of central nervous system, might occur (*3,4*). Transplacental infection of offspring that occurred during 2011 led to economic losses in animal husbandry of sheep, goats, and cattle during birthing periods occurring during November 2011 through spring 2012 (*5,6*).

Several vectors of SBV have been identified. Biting midges, small flying insects of the species *Culicoides*, were vectors for serotype 8 of bluetongue virus that emerged during 2006 in Europe (*7*), and they seem to play a key role in spreading SBV (*8,9*). Similar to distribution of serotype 8 of bluetongue virus in 2007 (*10*), SBV circulation occurred during 2012 in regions where viral circulation was limited or not yet detected in 2011 (*11*). However, few investigations of acute viral circulation in regions where most of the ruminant livestock were infected during 2011 have been performed. The high in-flock seroprevalence ranging from 70–100% in regions documenting SBV outbreaks (i.e., North Rhine-Westphalia in Germany, the Eastern part of Belgium, and the southern part of the Netherlands) is believed to limit reemergence of SBV (*12*).

The objective of this study was to assess whether SBV reemergence occurred in a sheep flock that had experienced an SBV infection outbreak during autumn 2011 and reached a seroconversion rate of 99.5% (*13*). Female lambs born in late autumn 2011 or early winter 2012 were followed bimonthly to assess natural SBV primary infection by using quantitative reverse transcription PCR (RT-qPCR) and seroneutralization (SN).

The Study

Sheep of a flock that belonged to the University of Namur that included ≈400 ewes (Ile de France, Laitier Belge, French Texel, and crossbred) and ≈20 rams were investigated. During the lambing period of January 2012, 28 (17%) of 163 newborn lambs showed signs of congenital SBV infection that was confirmed by real-time RT-qPCR and SN assay (*13*). Two SBV strains were isolated in cell culture of brain tissue samples from congenitally infected lambs (*14*). Retrospective analysis of sentinels' serum samples collected monthly in 2011 indicated that SBV infection of the flock occurred after September 15, 2011. SN assays were performed for all animals (n = 450) of the flock in January 2012 and revealed a seroprevalence of 99.5% (*13*).

Fifty female lambs born in autumn 2011 (n = 38) and January 2012 (n = 12) that were kept for breeding purposes were investigated by analyzing bimonthly blood samples collected during April–October 2012. The investigation protocol was approved by the Ethical Committee for Animal Welfare of the University of Namur (project 12/185). The animals were kept on pasture and underwent daily visual inspection. Blood was collected bimonthly and serum samples were analyzed by real-time RT-qPCR developed at the Friedrich Loeffler Institute (*1*) and by SN assay. RT-qPCR was performed on total RNA extracted from serum stored at –80°C. SN was performed after overnight incubation of a series of 2-fold dilutions for each serum sample with 100–200 tissue culture infectious $dose_{50}$ of SBV (isolate SBV-BH80/11–4). Virus back titration was performed. The data were respectively expressed as cycle threshold (C_t) and $\log_2$ 50% effective dose values. Results were considered positive at C_t<40 and $\log_2$ 50% effective dose >3.5.

As shown in Figure 1, the SBV infection of the sentinels evidenced by RT-qPCR was first detected around mid-July and ended in mid-October 2012. Before this period, RT-qPCR results were negative and decrease of colostrum derived antibodies was observed (data not shown). Few animals were infected in July (3 animals were positive by using RT-qPCR on July 27) and at the beginning of August. Of the positive RT-qPCR results, 80% were found between mid-August and late September. By October 17, ≥1 positive

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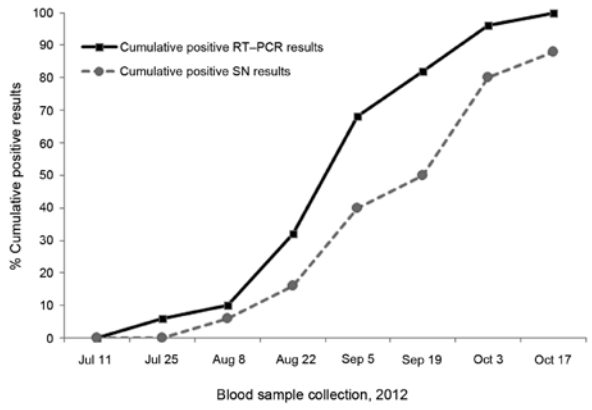

Figure 1. Time course of Schmallenberg virus spread among 50 infection-naive female lambs assessed bimonthly by real-time quantitative reverse transcription PCR (RT-qPCR) and seroneutralization (SN). Cumulative positive results (cycle threshold <40 and $\log_2$ 50% effective dose >3.5) obtained during July–October 2012 are expressed as percentages.

RT-qPCR result had been detected for each animal (median C_t 37.03; minimum 29.4, maximum 39.5). SN assay revealed positive results for SBV antibody titers within 2–4 weeks after infection evidenced by RT-qPCR results. These findings are similar to observations made after experimental and natural SBV infection in cattle (*1,10*). During the period of monitoring, no clinical signs were detected.

Against all expectations, all animals were positive for SBV at least once by using RT-qPCR. Ten lambs (20% of the investigated population) tested positive at 2-week intervals (Figure 2). This unexpected finding indicates that the duration of viremia in sheep (assessed as positive RT-qPCR result) may be longer after natural SBV infection in comparison to experimental SBV infection in cattle (*1*).

Although further investigations of a larger number of animals are needed, these preliminary results suggest that the time course of SBV infection depends on the age of the lambs (Table). The onset of SBV infection among lambs born in January 2012 occurred with a delay of 1 month. This finding is likely to be caused by the decrease of colostral protection.

The data collected during this study demonstrate that, even in regions with a high SBV seroprevalence, viral circulation and primary infection of naive animals occur. In addition to the question of duration of colostral protection, this observation raises questions about virus hosts. It might be hypothesized that other species are reservoirs. In autumn 2011, a seroprevalence of ≈43% was found in deer in the province of Namur (*15*), suggesting that deer might have been a reservoir during 2011–2012. Serum samples of 3 roe deer hunted in autumn 2012 in the forest near the sheep flock observed in this study tested positive by SN assay (N. Kirschvink, pers. comm.), indicating a higher percentage of seroconversion among samples from deer in 2012. It might further be speculated that notable vector activity favored viral circulation. During the summer 2007 serotype 8 of bluetongue virus outbreak, vector activity was assessed among the study flock of sheep and revealed substantial numbers of newly emerged female *Culicoides* spp. midges (*7*). This peak activity of vectors paralleled the episode of clinical serotype 8 of bluetongue virus manifestation. Hypothesizing that the local *Culicoides* spp. midge population did not substantially change over years, the intense infection rate during August–September 2012 could be related to increased seasonal vector activity.

Although reemergence of SBV among cattle is of concern, reemergence of SBV among sheep and goats is of particular importance, because high prolificacy and

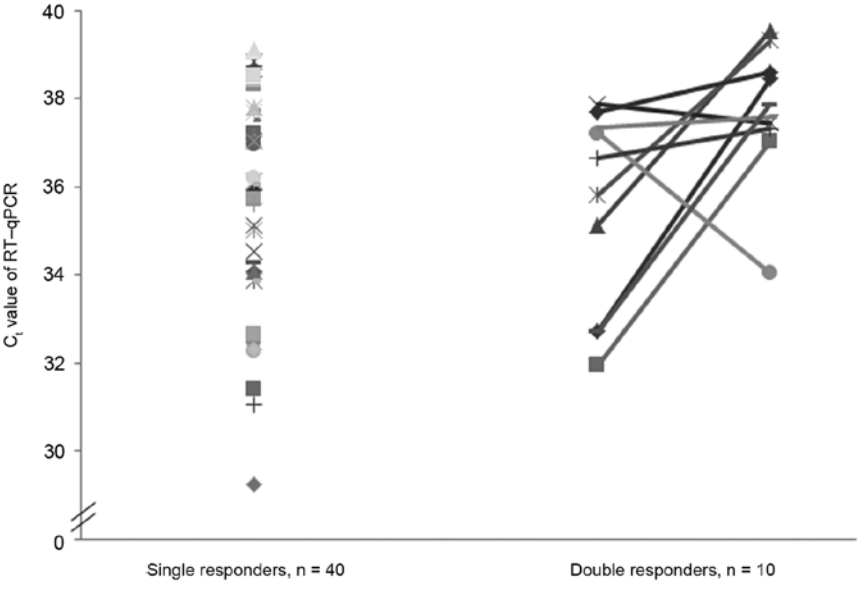

Figure 2. Individual cycle threshold (C_t) values of real-time quantitative reverse transcription PCR (RT-qPCR) in 50 female lambs at time of natural infection by Schmallenberg virus. C_t values of lambs that tested positive once (single responders, n = 40) and those of lambs that tested positive twice during a 2-week interval (double responders, n = 10) are shown. Each symbol represents 1 lamb.

Table: Time course of Schmallenberg virus spreading among female lambs born in late autumn and in January 2013 assessed by RT-q PCR and seroneutralization *

	2012							
Date of blood sampling	Jul 11	Jul 25	Aug 8	Aug 22	Sep 5	Sep 17	Oct 3	Oct 17
	No. (%) cumulative positive RT-qPCR results at each sampling date							
Lambs born in autumn 2012†	0	3 (8)	5 (13)	11 (29)	25 (66)	30 (79)	36 (95)	38 (100)
Lambs born in January 2013‡	0	0	0	5 (42)	9 (75)	11 (92)	12 (100)	12 (100)
	No. (%) cumulated positive seroneutralization results at each sampling date							
Lambs born in autumn 2012†	0	0	3 (8)	8 (21)	18 (47)	22 (58)	33 (87)	35 (92)
Lambs born in January 2013‡	0	0	0	0	2 (17)	2 (17)	6 (50)	8(67)

*Cumulated positive results obtained between July and October 2012 are expressed as absolute numbers and percentages.RT-qPCR results were considered positive if cycle threshold value was <40. Seroneutralization results were considered positive if $\log_2$ 50% effective dose values were > 3.5. RT-qPCR,real-time reverse transcription quantitative PCR.
†n=38.
‡n=12.

intensive reproduction of sheep and goats continuously lead to a noteworthy percentage of susceptible animals among the population. Moreover, the early onset of puberty in these species increases the probability of SBV infection at an early stage of gestation, leading to potential virus overwintering by transplacental infection of offspring, which could potentially lead to economic loss related to death or culling of offspring. Detailed knowledge about duration of viremia after natural SBV infection and duration of colostral protection are necessary for elaboration of efficient breeding and vaccination strategies.

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Mr Claine is a veterinary PhD student in the Veterinary Integrated Research Unit at University of Namur and investigates the impact of Schmallenberg virus infection on the unit's research sheep flock.

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Psychrobacter arenosus Bacteremia after Blood Transfusion, France

Yvan Caspar, Christine Recule, Patricia Pouzol, Bruno Lafeuillade, Marie-Reine Mallaret, Max Maurin, and Jacques Croize

We report a case of transfusion-associated bacteremia caused by *Psychrobacter arenosus*. This psychrotolerant bacterium was previously isolated in 2004 from coastal sea ice and sediments in the Sea of Japan, but not from humans. *P. arenosus* should be considered a psychrotolerant bacterial species that can cause transfusion-transmitted bacterial infections.

Bacteria are the leading cause of transfusion-transmitted infections (*1*). Contamination occurs more frequently in platelet concentrates than in erythrocyte units, especially because of different storage conditions (20°C–24°C for platelet concentrates vs. 1°C–6°C for erythrocyte units). However, several bacterial species are able to grow at 4°C (*1*–*3*). We report a case of transfusion-transmitted bacterial infection caused by *Psychrobacter arenosus*, an environmental psychrotolerant and halotolerant bacterium.

The Patient

In October 2009, a 58-year-old man was admitted to Grenoble University Hospital (Grenoble, France) for a blood transfusion because of severe anemia. Idiopathic medullary aplasia had been diagnosed in 1997, and he had had grade 3 myelofibrosis since 2006. He had been receiving palliative care since November 2007, and received transfusions of erythrocyte units every 3 weeks. On October 27, 2009, he received 3 erythrocyte units (at 8:30 AM, 10:30 AM, and 12:15 PM). While receiving the third unit, he became febrile (temperature of 38°C that rapidly increased to 40°C) and had chills and headache. The transfusion was stopped and the patient transferred to the Department of Internal Medicine. At examination, there was no hypotension, jaundice, or red urine.

Standard laboratory testing showed no ABO incompatibility, hemoglobinemia, hemoglobinuria, and coagulation disorders. According to recommendations of the Agence Nationale de Sécurité du Médicament (Saint-Denis, France), 3 sets of aerobic and anaerobic blood cultures (Bactec; Becton Dickinson, Pont de Clay, France) for the recipient (1 immediately and 2 others 4 hours later) and the remaining part of the third erythrocyte unit were sent to the bacteriology laboratory for culture. Gram staining of a blood smear prepared from the third erythrocyte unit showed a large number (≈10^6 CFU/mL) of gram-variable coccobacilli.

Samples were placed on Columbia blood agar (bioMérieux, Marcy L'Etoile, France) and incubated at 37°C in anaerobic or 5% CO_2–enriched atmospheres. Sample inoculated into blood culture bottles were incubated at 37°C under aerobic and anaerobic conditions (Figure). The aerobic blood culture bottle of the first sample obtained from the recipient and aerobic cultures of the third erythrocyte unit enabled isolation of the same gram-variable coccobacilli after incubation for 48 hours (Figure). Colonies obtained on Columbia blood agar were monomorphic, small, and gray, and had positive results for oxidase and catalase tests. Phenotypic traits of the bacterial strains isolated from the blood of the patient and the erythrocyte unit were similar, but identification using the Vitek2 Gram negative card and API 20E, API 20NE, and ID 32 GN Kits (bioMérieux) was not successful.

Molecular identification was performed by 16S rRNA gene amplification and sequencing with fD1 and rP2 primers (*4*), and DNA sequence analysis was performed by using BLAST (www.ncbi.nlm.nih.gov) and leBIBI (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi) software. DNA sequences obtained were identical (Genbank accession no. JX416703) and showed 99.7% homology with the *P. arenosus* 16SrDNA sequence previously reported by Romanenko et al. (*5*) (Genbank accession no. AJ609273). Consistent with this identification, subcultures of the isolated strain obtained on tryptic soy agar plates incubated at 4°C, 25°C, and 37°C showed opaque, circular, convex, cream-colored colonies; no subcultures were obtained on Drigalski medium. Phenotypic characteristics of this strain and the strain isolated by Romanenko at al. (*5*) are summarized in the Table. To determine the source of the *P. arenosus* contamination, environmental samples were collected at sites in which erythrocyte units were prepared and stored, but culture results were negative.

Antimicrobial drug susceptibility was determined by using an agar disk diffusion method, and results were

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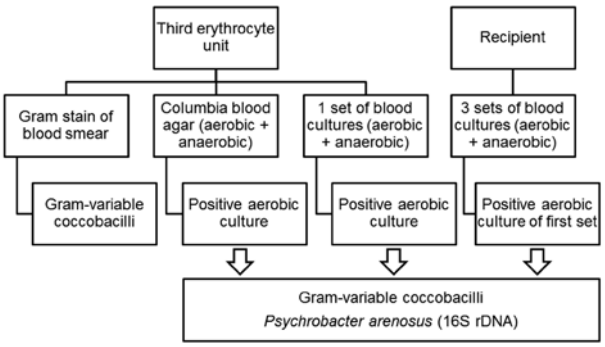

Figure. Flow diagram showing samples collected from the blood donor unit (third erythrocyte unit) and a 58-year-old man (transfusion recipient) and results for isolation and identification of *Psychrobacter arenosus*, France.

interpreted by using MIC breakpoints recommended for other oxidative gram-negative bacilli by the Comité de l'Antibiogramme de la Société Française de Microbiologie (Paris, France) (*6*). The isolate was resistant to lincomycin and susceptible to amoxicillin, amoxicillin/clavulanate, ticarcillin/clavulanate, piperacillin, piperacillin/tazobactam, cefalotin, cefotaxime, ceftazidime, cefpirome, cefepime, imipenem, gentamicin, tobramycin, netilmicin, amikacin, erythromycin, pristinamycin, polymyxin B, trimethoprim/sulfamethoxazole, nalidixic acid, ofloxacin, ciprofloxacin, and fosfomycin.

The patient initially received intravenous ticarcillin/clavulanate (5 g/200 mg, 3×/d) and vancomycin (1g, 2×/d). When the antibiogram was available, treatment was switched to oral administration of amoxicillin/clavulanate (1 g/125 mg, 3×/d) and ofloxacin (200 mg, twice a day) for 12 days, which resulted in rapid recovery.

Conclusions

Psychrobacter species are nonmotile, nonpigmented, aerobic, gram-negative coccobacilli, although Gram staining results are often variable (*7*). These bacteria are psychrotolerant and halotolerant environmental microorganisms (*7*). They have been isolated from many sources, including sea water, ornithogenic soil, air contaminants, fish, poultry, milk, cheese, and irradiated food (*7*). *P. arenosus* was isolated in 2004 from coastal sea ice and sediments in the Sea of Japan (*5*).

Psychrobacter species are considered rare opportunistic human pathogens (*8*) and have been isolated from specimens obtained from human blood, cerebrospinal fluid, brain tissue, urine, ears, eyes, vulvae, wounds, and other cutaneous sources (*8*,*9*). *P. phenylpyruvicus* (formerly *Moraxella phenylpyruvica*) has been associated with bacteremia, endocarditis, septic arthritis, foot abscess, and surgical wound infection (*9–11*). *P. immobilis* has caused fatal infections in a patient who had AIDS (*12*), nosocomial ocular infection (*13*), and meningitidis in a 2-day-old infant (*14*). However, recently the taxonomy of *Psychrobacter* species has been revised, and most human isolates other than *P. phenylpyruvicus* belong to the newly characterized species *P. faecalis* and *P. pulmonis* (*8*). Also, a novel species, *P. sanguinis,* has been isolated from human blood samples (*15*). Thus, the spectrum of human infections associated with the different species of the genus *Psychrobacter* could change rapidly.

Table. Characteristics of *Psychrobacter arenosus* isolated in this study (France) and a strain isolated in Russia*

Characteristic	Isolate from this study	Isolate from Russia†
Growth at 5°C	+	+
Growth at 22°C	+	+
Growth at 37°C	+	+
Nitrate reduction	–	–
Urease	–	–
Arginine dihydrolase	–	–
β-galactosidase	–	–
Esculin hydrolysis	–	–
Gelatinase	–	–
Indole production	–	–
Metabolic assay result		
L-arabinose	–	+
Malate	+	+
Citrate	+	+
Caprate	–	–
Acetate	+	+
Propionate	+	+
3-hydroxybutyrate	+	–
Lactate	+	–
Itaconic acid	+	–
L-proline	+	–
L-alanine	+	–
L histidine	+	–
L-serine	+	UNK
Valeric acid	+	UNK
Adipic acid	–	UNK
3-hydroxybenzoate	–	UNK
4-hydroxybenzoate	–	UNK
L-fucose	–	UNK
Gluconate	–	UNK
2-ketogluconate	–	UNK
N-acetylglucosamine	–	UNK
D-glucose	–	UNK
Glycogen	–	UNK
Inositol	–	UNK
Malonate	–	UNK
D-maltose	–	UNK
D-mannitol	–	UNK
D-melibiose	–	UNK
D-mannose	–	UNK
Phenylacetate	–	UNK
L-rhamnose	–	UNK
D-ribose	–	UNK
D-saccharose	–	UNK
Salicin	–	UNK
D-sorbitol	–	UNK
Suberic acid	–	UNK

*+, positive; –, negative; UNK, unknown.
†Romanenko et al. (*5*).

We report a case of human moderate septic transfusion reaction caused by *P. arenosus*. The clinical and laboratory findings did not support an acute hemolytic transfusion reaction. Gram staining of a direct smear prepared from the erythrocyte unit showed a high bacterial inoculum, strongly suggesting multiplication of bacteria in this unit before transfusion. *P. arenosus* was isolated from a contaminated erythrocyte unit and blood of the patient obtained after the transfusion was stopped. The patient recovered rapidly after receiving appropriate antimicrobial drug therapy. These findings confirm that the transfusion reaction was attributable to *P. arenosus* contamination of the erythrocyte unit. However, the isolated strain was not identified until 16S rRNA gene amplification and sequencing were performed.

We found differences in biochemical characteristics between this *P. arenosus* strain and the strain isolated by Romanenko et al. (*5*) (Table). *P. arenosus* is able to grow at 4°C–37°C (*5*) and thus could multiply in the erythrocyte unit stored at 4°C for 1 month before transfusion. As in most cases of transfusion-transmitted bacterial infections, source of contamination of the erythrocyte unit was not identified. *P. arenosus* could not be detected in environmental samples collected at sites in which the erythrocyte unit was prepared and stored. As for other gram-negative bacteria, transient bacteremia in an asymptomatic blood donor could be the source of the erythrocyte unit contamination (*1*,*3*), but exogenous contamination at the time of blood collection or preparation of units occurs more frequently (*3*).

Psychrobacter spp. strains are highly susceptible to antimicrobial drugs; only 1 strain of *P. phenylpyruvicus* was reported to be resistant to penicillin and aztreonam, 2 strains of *P. immobilis* resistant to penicillin (*10*,*13*,*14*), and 1 strain of *P. immobilis* resistant to gentamicin, tobramycin, ampicillin, and lincomycin (*12*). Most human infections have been treated with a third-generation cephalosporin, leading to rapid recovery (*10*,*11*,*14*). One patient who had AIDS died from septic shock, despite appropriate treatment (*12*).

In conclusion, *P. arenosus* should be considered a psychrotolerant bacterial species responsible for transfusion-transmitted bacterial infections, similar to *Yersinia enterocolitica*, *Listeria monocytogenes*, and psychrophilic *Pseudomonas* spp. (*1,2*). However, phenotypic identification of *P. arenosus* is problematic and might require amplification and sequencing of its 16S rRNA gene.

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Ciprofloxacin-Resistant *Campylobacter* spp. in Retail Chicken, Western Canada

Agnes Agunos, David Léger, Brent P. Avery, E. Jane Parmley, Anne Deckert, Carolee A. Carson, and Lucie Dutil,

During 2005–2010, the Canadian Integrated Program for Antimicrobial Resistance Surveillance identified increased prevalence of ciprofloxacin (a fluoroquinolone) resistance among *Campylobacter* isolates from retail chicken in British Columbia (4%–17%) and Saskatchewan (6%–11%), Canada. Fluoroquinolones are critically important to human medicine and are not labeled for use in poultry in Canada.

Human campylobacteriosis, a notifiable disease in Canada, is the most common cause of bacterial enteric infections among persons in Canada; in 2005, the incidence rate of campylobacteriosis was 30.9 cases per 100,000 population (*1*). In chickens, *Campylobacter* spp. are not clinically relevant; however, the presence of these bacteria in poultry represents a potential threat to public health (*2*).

Ciprofloxacin, a fluoroquinolone antimicrobial drug, is indicated for the treatment of respiratory, urinary, skin, and bone/joint infections and gastroenteritis in adults (*3*). In 2008 in Canada, fluoroquinolones were the fourth most frequently dispensed class of antimicrobial drug (dispensed for oral use by retail pharmacists; www.phac-aspc.gc.ca/cipars-picra/2008/4-eng.php#Hum0). A study investigating antimicrobial drug use and resistance in 2 health units in Ontario found that ciprofloxacin was the antimicrobial drug most frequently used to treat human campylobacteriosis (*4*). Fluoroquinolones are considered "critically" or "very" important to human medicine by the World Health Organization (*5*) and the Veterinary Drugs Directorate (VDD), Health Canada (*6*). The veterinary fluoroquinolones enrofloxacin and danofloxacin are VDD Category I antimicrobial drugs labeled for use in companion animals and beef cattle, but they are not labeled for use in poultry. The VDD has established a policy recommending against the extra-label use of Category I antimicrobial drugs in food-producing animals (*7*); however, Canada does not have legislation restricting this extra-label use.

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The Study

The Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS), Public Health Agency of Canada, collects samples of fresh chicken, beef, and pork from retail outlets in British Columbia, Saskatchewan, Ontario, Québec, and Maritimes (New Brunswick, Nova Scotia, and Prince Edward Island) on a routine basis and cultures them for select bacteria. Retail food samples best reflect the level of consumer exposure to drug-resistant foodborne bacteria. Methods used for sample collection, culture, and antimicrobial drug-susceptibility testing are described in the CIPARS annual reports (www.phac-aspc.gc.ca/cipars-picra/2008/6-eng.php#Ant).

Up to and including 2010, *Campylobacter* spp. were isolated from retail chicken meat samples in the provinces of British Columbia (since 2007, 225 isolates/536 samples [42%]), Saskatchewan (since 2005, 276/884 [31%]), Ontario (since 2003, 845/2288 [37%]), Québec (since 2003, 683/2215 [31%]), and the Maritimes (since 2008, 117/444 [26%]) (Table). Temporal trends in ciprofloxacin resistance among *Campylobacter* spp. isolates are shown in the Figure. Since initiation of surveillance, prevalence of ciprofloxacin resistance has significantly increased in British Columbia and Saskatchewan. The highest recovery of ciprofloxacin-resistant *Campylobacter* spp. was in British Columbia in 2009 (28.6%, 22/77), when recovery of all *Campylobacter* spp. (52.7%, 77/146) was also highest.

Significant differences in prevalence between 2 periods were assessed by using χ^2 tests. The increased trend in British Columbia seems more abrupt, especially from 2008 to 2009, relative to Saskatchewan, where the increase is relatively more gradual. Combined data from 2009–2010 showed that 23% (34/147) of *Campylobacter* spp. isolates from British Columbia and 13% (11/84) of isolates from Saskatchewan were resistant to ciprofloxacin, compared with 6% (5/78, $p = 0.002$) and 6% (11/192, $p = 0.04$) of isolates resistant to ciprofloxacin in combined data before 2009 in British Columbia (2007–2008) and Saskatchewan (2005–2008), respectively. By comparison, in 2009–2010, the overall prevalence of ciprofloxacin-resistant *Campylobacter* spp. from retail chicken collected in Ontario, Québec, and the Maritimes has remained <3% (10/395), significantly lower ($p<0.001$) than the overall prevalence in British Columbia and Saskatchewan (19%, 45/231). The current prevalence of ciprofloxacin resistance in British Columbia and Saskatchewan is also higher than that reported by studies of antimicrobial

Table. *Campylobacter* spp. isolated from retail chicken in Canada, by province, 2003–2010*

Province	2003	2004	2005	2006	2007	2008	2009	2010
British Columbia								
Samples collected, no.					80	145	146	165
Campylobacter spp. isolates, no. (%)					28 (35.0)	50 (34.5)	77 (52.7)	70 (42.4)
Ciprofloxacin-resistant isolates, no. (%)					1 (3.6)	4 (8.0)	22 (28.6)	12 (17.1)
Saskatchewan								
Samples collected, no.			145	155	141	161	150	132
Campylobacter spp. isolates, no. (%)			52 (35.9)	51 (32.9)	49 (34.8)	40 (24.8)	48 (32.0)	36 (27.3)
Ciprofloxacin-resistant isolates, no. (%)			3 (5.8)	1 (2.0)	3 (6.1)	4 (10)	7 (14.6)	4 (11.1)
Ontario								
Samples collected, no.	166	316	303	312	320	311	328	232
Campylobacter spp. isolates, no. (%)	78 (47.0)	140 (44.3)	120 (39.6)	105 (33.7)	117 (36.6)	120 (38.6)	101 (30.8)	64 (27.6)
Ciprofloxacin-resistant isolates, no. (%)	3 (3.8)	3 (2.1)	3 (2.5)	3 (2.9)	1 (0.9)	5 (4.2)	1 (1.0)	3 (4.7)
Québec								
Samples collected, no.	170	322	299	288	287	287	266	296
Campylobacter spp. isolates, no. (%)	94 (55.3)	158 (49.1)	103 (34.4)	100 (34.7)	59 (20.6)	54 (18.8)	52 (19.5)	63 (21.3)
Ciprofloxacin-resistant isolates, no. (%)	3 (3.2)	4 (2.5)	2 (1.9)	2 (2.0)	8 (13.6)	0	0	1 (1.6)
Maritimes								
Samples collected, no.						55	199	190
Campylobacter spp. isolates, no. (%)						2 (3.6)	47 (23.6)	68 (35.8)
Ciprofloxacin-resistant isolates, no. (%)						0	2 (4.3)	3 (4.4)

*Blank cells indicate not applicable (no samples collected or tested).

drug resistance in *Campylobacter* spp. in Ontario (*8*) and Québec (*9*).

Several factors contributing to the emergence of ciprofloxacin resistance have been hypothesized and include antimicrobial drug use in broiler breeder and broiler chickens and importation of poultry products. Data for antimicrobial drug use in the poultry industry are not currently available. Fluoroquinolone use can select for ciprofloxacin resistance and result in the emergence and persistence of resistant *Campylobacter* spp. (*10*). Evidence indicates vertical transmission of fluoroquinolone-resistant *Campylobacter* spp. (*11*). Surveillance data from the United States showed the persistence of ciprofloxacin resistance, despite a 2005 ban on fluoroquinolone use in chickens (*12*). Ciprofloxacin resistance also persisted in broilers raised in Denmark after fluoroquinolone use decreased in 2006 (*13*). Current use of ciprofloxacin in US poultry is unknown. However, in Denmark, use in few broiler breeders has been reported (*13*). It is plausible that fluoroquinolone use in breeder and broiler chickens could explain the generation and maintenance of ciprofloxacin resistance.

Another antimicrobial drug–use practice might play a role in ciprofloxacin resistance. Tetracyclines are labeled for use in broiler chickens. Across the regions sampled by CIPARS, a high proportion (64%) of ciprofloxacin-resistant *Campylobacter* isolates were also resistant to tetracycline. Although the prevalence rates for ciprofloxacin and tetracycline resistance were quite different, the resistance trends in British Columbia and Saskatchewan were strikingly similar in shape. Tetracycline resistance was significantly associated with ciprofloxacin resistance in isolates from British Columbia and Saskatchewan (odds

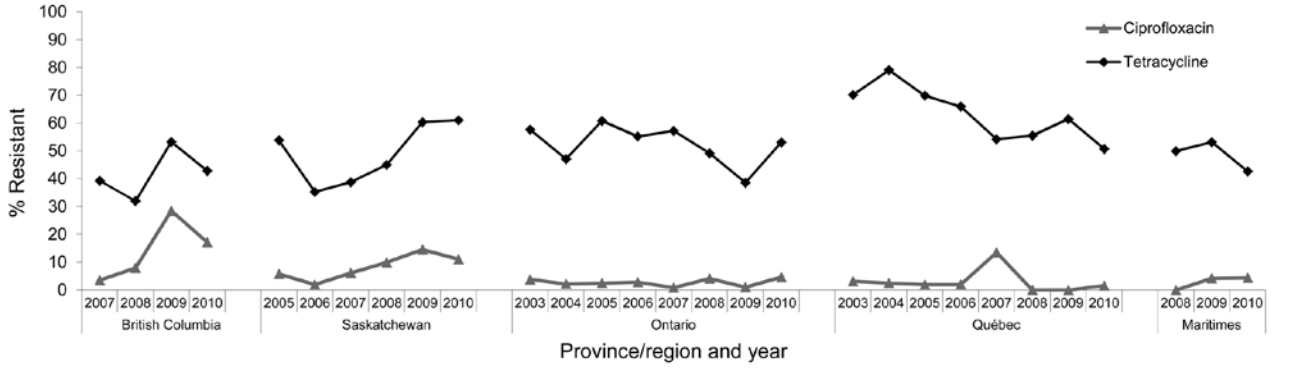

Figure. Temporal trends in ciprofloxacin and tetracycline resistance among *Campylobacter* isolates from chicken, Canada, 2003–2010. Data for 2003 to 2009 were published in the CIPARS 2009 Preliminary Report (www.phac-aspc.gc.ca/cipars-picra/2009/1-eng.php#fig_21). Data for 2010 were published in the CIPARS 2010 Short Report (can be requested at www.phac-aspc.gc.ca/cipars-picra/pubs-eng.php). CIPARS, Canadian Integrated Program for Antimicrobial Resistance Surveillance, Public Health Agency of Canada.

ratio 3.04; 95% CI 1.70–5.48; p<0.001) but not in those from the eastern part of the country (odds ratio 0.99; 95% CI 0.54–1.38; p = 0.951). A similar phenomenon has recently been noted among *Campylobacter* spp. recovered from domestically raised and imported broiler chickens in Denmark (*13*). We currently have no explanation for this observation; *gyrA* mutation and overexpression of the multidrug-resistant efflux pump *CmeABC* (*14*), as a result of exposure to ciprofloxacin and ciprofloxacin/tetracycline, respectively, might have played a role. Molecular investigations of organisms isolated from retail chicken by CIPARS have yet to be conducted.

Another factor to consider is the importation of poultry products. CIPARS collects raw, fresh (never frozen) chicken primarily produced and distributed in Canada. It is possible that a small proportion of the fresh retail meat sampled originated in other countries, but the volume of imported meat (≈75 tonnes [167 million pounds] or 7.5% of the previous year's production; [*15*]) is unlikely to explain the regional increase in *Campylobacter* spp. resistance. Furthermore, importation of meat is not restricted to only British Columbia and Saskatchewan (www.international.gc.ca/controls-controles/prod/agri/chicken-poulet/index.aspx?menu_id = 26&view = d). According to trade agreements, ≈141 million chicks/eggs (≈21.1% of the previous year's grown broiler production) are imported annually from the United States. These imported broiler chicks/eggs might be an additional source of drug-resistant subtype introduction into the Canadian poultry production system. To evaluate the prevalence of resistance in domestically raised flocks, the CIPARS abattoir component added *Campylobacter* spp. surveillance in chickens in 2010. From this surveillance, 4/111(4%) isolates from domestically raised chicken ceca were resistant to ciprofloxacin, 3 from British Columbia, and 1 from the Maritimes, confirming the potential domestic origin of these strains in retail chicken.

Conclusion

The Public Health Agency of Canada is concerned by the emergence of resistance to ciprofloxacin, which is critically important for treatment of *Campylobacter* spp. infection in humans. Extra-label use of fluoroquinolones in the broiler breeder or broiler chicken sectors might have contributed to the emergence of this resistance. The role of importation of poultry products as a potential source of resistant strains requires further investigation. The broiler industry is collaborating with CIPARS to create a farm surveillance program, which would capture data on antimicrobial drug use and resistance.

Veterinary fluoroquinolones are not labeled for use in poultry in Canada. The VDD policy recommends against the extra-label use of Category I antimicrobial drugs in food-producing animals. More research is required to assess mechanisms responsible for the trends observed for tetracycline and ciprofloxacin resistance. Furthermore, genotyping of isolates from humans and chickens (retail and/or abattoir) from British Columbia and Saskatchewan is required to determine if strains are epidemiologically related.

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Asynchronous Onset of Clinical Disease in BSE-Infected Macaques

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To estimate the effect of the variability of prion disease onset on primary bovine spongiform encephalopathy transmission to humans, we studied 6 cynomolgus macaques. The preclinical incubation period was significantly prolonged in 2 animals, implying that onset of variant Creutzfeldt-Jacob disease in humans could be more diverse than previously expected.

Prion diseases, such as bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep, and Creutzfeldt-Jakob disease (CJD) in humans, are fatal, transmissible, neurodegenerative disorders associated with the aggregation of an infectivity-associated isoform (PrP^{Sc}) of the cellular prion protein (PrP) (*1*). Seventeen years ago, it became apparent that the BSE-infectious agent had entered the food chain and was identified as the causative agent for a new variant CJD (vCJD) (*2*). Since then, several risk assessment studies have investigated the number of expected vCJD cases in human populations (reviewed in [*3*]). Although thousands to millions of consumers of beef products were estimated to be affected, thus far only a few more than 200 vCJD cases have been observed worldwide.

This discrepancy was assumed to be attributable to the so-called species barrier, defined as the hindrance of an infectious agent to change its natural host. Upon crossing the species barrier, prion diseases often show a low attack rate in conjunction with a high variability in the preclinical incubation time. Thus, the consumption of BSE-contaminated products may have led either to a restricted infection or to a prolonged asymptomatic phase in some exposed persons. Therefore, concerns have been raised that asymptomatic carriers of vCJD might exist, posing a risk for unintentional human-to-human transmission.

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First indications that transmission of BSE to primates may lead to variances in the preclinical incubation times were obtained by inoculating cynomolgus macaques with cattle-derived BSE material (*4*–*6*), even though in those studies not more than 3 animals were used. We have now used a group of 6 macaques that were infected with BSE at a comparable age and kept under identical and controlled experimental conditions.

The Study

Six captive-bred female cynomolgus macaques (*Macaca fascicularis*, purchased from the Centre de Recherche en Primatologie, Mauritius) were inoculated intracerebrally with 1 dose of 50 mg brain homogenate (10% wt/vol) derived from 11 BSE-infected cattle. Animal experimentation was performed in accordance with section 8 of the German Animal Protection Law in compliance with Directive 86/609/EEC. Macaques were housed in a social group, and behavioral changes were assessed on a daily basis by experienced animal care takers.

After inoculation, all 6 macaques remained healthy and asymptomatic for >30 months (Table). At 931 days postinfection, 1 animal showed indications of slight coordination disorders. Within a few days, afferent ataxia developed, and when the animal was separated from the others animals, she apparently became tame. After 2 weeks, the animal showed severe dysmetria of the extremities without obvious myoclonia. Dementia was apparent but could not be diagnosed by objective measures. For ethical reasons, the animal was euthanized 17 days after disease onset. Within the next 14 weeks, 3 more animals became symptomatic. After appearance of neurologic symptoms (ataxia, tremors), the affected animals were occasionally separated from the group when symptoms became more severe or attacks from asymptomatic animals occurred. The disease course in these animals was comparable to that of the first animal, but the progression was slower (91–103 days).

Two of the 6 animals remained asymptomatic for ≈1 additional year. Although daily monitoring was facilitated by the fact that only 2 macaques remained and that the caretakers were more experienced to recognize minor changes in behavior, symptoms were first detected 1,340 and 1,398 days postinfection, respectively. Clinical signs were similar to those observed in the previous 4 animals. The symptomatic periods before euthanasia for these macaques lasted 103 and 143 days, respectively (Table). Direct comparison revealed that the difference between the short (931–1,025 days) and the long (1,340–1,398 days) preclinical incubation time was statistically significant (Figure 1, log-rank [Mantel-Cox] test, $p<0.05$).

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Table. Incubation periods of cynomolgus macaques infected intracerebrally with 50 mg brain homogenate from bovine spongiform encephalopathy–infected cattle*

Animal	Haplotype at codon 129	First clinical signs, dpi	Duration of clinical phase, dpi
A1	M/M	931	17
A2	M/M	1,398	103
A3	M/M	946	91
A4	M/M	1,025	94
A5	M/M	1,340	143
A6	M/M	946	103

*dpi, days postinfection.

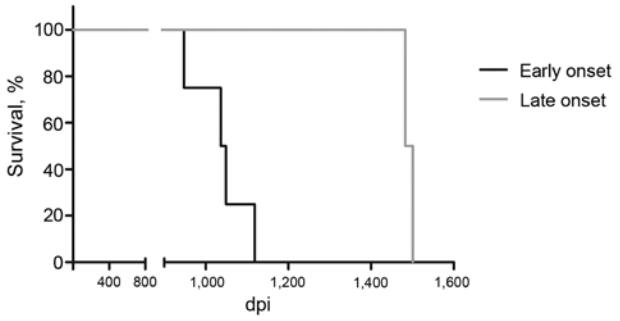

Figure 1. Survival of intracerebrally BSE-infected cynomolgus macaques. Six age- and sex-matched cynomolgus macaques were inoculated intracerebrally with 50 mg brain homogenate (10% in sucrose) derived from 11 BSE-infected cattle. Macaques were euthanized at severe signs of neurodegenerative disease. The animals were grouped according to early (<1,200 dpi, n = 4) or late (>1,200 dpi, n = 2) onset of disease. The respective survival curves were compared by using a log-rank test (Mantel-Cox, $p<0.05$). BSE, bovine spongiform encephalopathy; dpi, days postinfection.

Test results of brain samples from all animals were positive for macaque-adapted BSE by Western blot analysis. In brief, brain tissue from each animal was homogenized and subjected to proteinase K (PK) treatment for 1 h at 37°C. Samples were separated on acrylamide gels and transferred to nitrocellulose membranes. Macaque-adapted BSE (PrP^{Sc}) was detected by using the monoclonal anti-PrP antibody 11C6. PK-resistant PrP was detected in all 6 macaques, confirming that BSE was transmitted to the animals.

The individual glycopattern and band migration of macaque-adapted PrP^{Sc} was compared with human sporadic CJD (sCJD) type 1, sCJD type 2, and vCJD. PK-resistant PrP from BSE-infected macaques co-migrated with type 2 sCJD and was clearly distinguishable from type 1 sCJD (Figure 2). The glycosylation pattern of macaque-adapted BSE was comparable with vCJD (*6*,*7*), which is characterized by an overrepresentation of diglycosylated PrP^{Sc} (*8*,*9*). Using 11C6 antibody (*10*), we detected a slightly decreased signal of the diglycosylated PrP^{Sc} isoform for sCJD, vCJD, and macaque-adapted BSE. We assume that this effect is related to a reduced affinity of the diglycosylated isoform to 11C6 that otherwise shows high sensitivity to macaque-adapted PrP^{Sc}. Nevertheless, direct comparison showed a higher amount of the diglysosylated PrP^{Sc} isoform in vCJD and macaque-adapted BSE than sCJD, which was also shown with a different monoclonal antibody, 3F4. This finding confirms that BSE transmission to macaques is comparable with, and can be used as a model for, human vCJD infection.

Conclusions

Several susceptibility studies using nonhuman primates as a model for human prion diseases hint to heterogeneity of the preclinical incubation period upon crossing the species barrier (*5*,*11*,*12*). However, because of the low number of no more than 3 animals, this variability was not always evident (*4*). Therefore, there was an urgent need to determine whether the transmission of BSE to humans is likely to lead to a similar diversity.

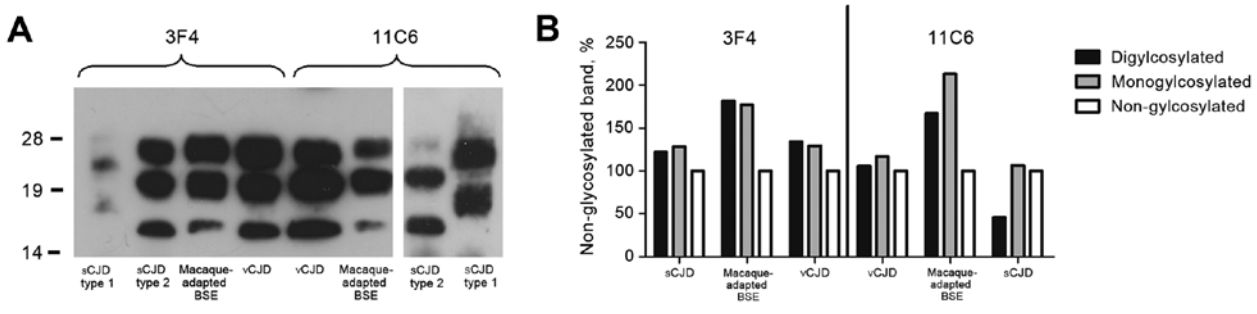

Figure 2. PrP^{Sc} profile of macaque-adapted BSE in comparison to human CJD. Brain homogenates from human sCJD type 1, sCJD type 2, vCJD, and BSE-infected macaques were subjected to PK treatment, separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. A) PrP^{Sc} for human and macaque brain was detected with the widely used monoclonal antibody 3F4 or with 11C6. B) Glycoform ratio of sCJD type 2, vCJD, and macaque-adapted BSE. The relative signal intensities of the diglycosylated, monoglycosylated, and nongylcosylated isoforms were determined densitometrically and normalized to the band of the nongylcosylated isoform. PrP^{Sc}, prion protein isoform; BSE, bovine spongiform encephalopathy; CJD, Creutzfeldt-Jakob disease; sCJD, sporadic CJD; vCJD, variant CJD; PK, proteinase K.

Our study using 6 cynomolgus macaques shows that the transmission of BSE to primates led to a significantly prolonged asymptomatic phase in 2 animals. Disease onset is influenced by several factors (*13*). Our study design enabled us to exclude that the route of transmission influenced the disease progression because the infectious agent was injected into the same brain region of each animal. Also, a limited infectious dose cannot be responsible, as shown by the attack rate of 100%. In addition, endogenous factors, such as age, the MM genotype at codon 129 (Table), and housing conditions, were comparable for all macaques.

Thus, we conclude that the variable asymptomatic phase is most likely influenced by the infectious agent (*14*) or the genomic diversity of the macaques (*13*). The animals in our study were not inbred. Therefore, differences in the genomic background may have influenced the time of disease onset. In contrast, the PrP^{Sc} migration patterns of the animals give no indications for different types or strains that evolved from the mixed BSE inoculum. However, further studies will have to verify this.

Nevertheless, during the BSE epidemics, the human population with its natural genomic diversity was also exposed to a nonhomogenous prion source. Therefore, our study closely mimics the human situation. Our results imply that a prolonged asymptomatic phase can be expected for vCJD. In light of the transmissibility of vCJD through blood transfusions (*15*), our findings emphasize the need for continued attention to the risks of secondary human-to-human transmission.

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Dr Montag is a microbiologist at the Department of Molecular and Cell Physiology at the Hannover Medical School. Her primary research interests are the molecular mechanisms of disease pathology, including prion disorders and inherited cardiac diseases.

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Prevalence of Nontuberculous Mycobacteria in Cystic Fibrosis Clinics, United Kingdom, 2009

Paul Seddon, Katy Fidler, Sundhya Raman, Hilary Wyatt, Gary Ruiz, Caroline Elston, Felicity Perrin, Khin Gyi, Diana Bilton, Francis Drobniewski, and Melanie Newport

Incidence of pulmonary infection with nontuberculous mycobacteria (NTM) is increasing among persons with cystic fibrosis (CF). We assessed prevalence and management in CF centers in the United Kingdom and found 5.0% of 3,805 adults and 3.3% of 3,317 children had recently been diagnosed with NTM. Of those, 44% of adults and 47% of children received treatment.

Nontuberculous mycobacteria (NTM) are ubiquitous environmental organisms (*1*), broadly classifiable into "slow" and "rapid" growers. Many species, especially the slow-growing *Mycobacterium avium* complex (MAC), are known to cause disseminated disease in immunodeficient persons (*2,3*). Rapid growers include the phylogenetically similar *M. abscessus* and *M. chelonae*, referred to as *M. abscessus* complex (MABSC). Such organisms have emerged as pathogens in immunocompetent adults, for example, after traumatic limb injuries sustained during the 2004 tsunami in the Indian Ocean (*4*). NTM have been identified as pulmonary pathogens in immunocompetent middle-aged women with nodular bronchiectasis (*5*) and older men who smoke with upper lobe cavitation (*6*). Since the 1990s, NTM have been increasingly isolated from the sputum of patients with cystic fibrosis (CF) (*7,8*).

CF is the most frequent lethal genetic disorder of White persons, affecting >8,000 persons in the United Kingdom and 30,000 in the United States. Early death is mainly from chronic lung disease caused by persistent lower airway infection and inflammation. Important airway pathogens include *Staphylococcus aureus* and *Pseudomonas aeruginosa*, but others, such as NTM, are playing an increasingly recognized role.

A multicenter prospective study of CF patients in the United States (*9*) found the prevalence of NTM in sputum to be 13%; MAC was the most common species (72%), and M. abscessus was the next most common (16%). Older age was the most significant predictor for a positive sputum culture. A multicenter CF study in France reported a prevalence of 6.6%, showing MABSC to be the most common; MAC was the next most prevalent (*10*). Single-center studies in Europe have found variable NTM prevalences, from 13.3% in a center in Germany (*7*) to 3.8% in a center in the United Kingdom (*8*).

It is often difficult to determine whether isolation of NTM represents colonization or disease that requires treatment. Current American Thoracic Society guidelines (*1*) are helpful, but application lacks uniformity, and management is complicated by inducible resistance to antibiotics (*11*).

To determine the optimal management of NTM in CF, we first need to define the extent of the problem and characterize current practice. The aim of this study was to establish the prevalence of NTM infection in the UK CF community, how it is screened for, and how it is managed.

The Study

A single-page questionnaire (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/12-0615-Techapp1.pdf) was sent to the lead CF physician in all UK pediatric and adult CF centers identified by the principal UK CF charity, the Cystic Fibrosis Trust, in 2009. Nonresponders were followed up with by email or telephone. The local research ethics committee deemed the study did not require ethical approval.

Results were tabulated and basic statistical analysis performed by using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA). Because *M. chelonae* and *M. abscessus* have common phenotypic characteristics and not all samples were fully sequenced by a reference laboratory, we combined these for reporting as MABSC.

Twenty-three adult and 29 pediatric UK CF centers, with 8,513 CF patients, were sent questionnaires. Responses were received from 19 (83%) and 24 (83%) of these centers respectively, comprising 7,122 patients (3,805 adults, 3,317 children), or 84% of the UK CF population. As expected, the prevalence of any NTM isolate was higher in the adult (5.0%) than in the pediatric (3.3%) population (Table). Fifteen adult and 17 pediatric centers cultured samples for NTM yearly; most remaining centers tested for NTM only if there was a specific clinical indication.

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Table, NTM isolation rate and screening frequency among adult and pediatric cystic fibrosis clinics, United Kingdom, 2009

CF clinic type	No. clinics receiving/no. responding to survey (%)	Total no. patients	No. (%) clinics		Frequency of requesting NTM culture
			With any NTM isolates in previous 2 y	With >2 NTM isolates in previous 2 y	
Adult	19/23 (83)	3,805	190 (5.0)	135 (3.6)	Yearly: 15 Every 6 months: 1 Every 3 months 1 When clinically indicated: 2
Pediatric	24/29 (83)	3,317	110 (3.3)	74 (2.2)	Yearly: 17 Every 6 months: 0 Every 3 months 0 When clinically indicated: 7

*NTM, nontuberculous mycobacteria.

The NTM pathogens most frequently cultured were the rapidly growing MABSC (62% of adults, 68% of children who were NTM-positive), followed by MAC (28% of adults, 27% of children). Other species (*M. gordonae, kansasii, xenopi, fortuitum, simiae, malmoense, mucogenicum, perigrinum*) together made up 8% of the total. Wide geographic variation was noted, increasing from northwest to southeast, manifesting lowest prevalence in Northern Ireland (1.9%) and highest in South East England (7.5%) (Figure).

Criteria for starting treatment varied widely between centers. Most centers required clear clinical or radiologic deterioration in addition to positive cultures, but a significantly higher proportion of adult centers (42%) than pediatric centers (21%) were prepared to commence treatment on the basis of repeated culture or smear positivity alone. Despite this, similar proportions of NTM-positive adults and children had received specific treatment for NTM in the 2 years preceding the survey: 44% of adults and 47% of children with any NTM isolate were treated, and 62% of adults and 70% of children in whom >2 isolates were found were treated. Six adults and 2 children who had CF had been refused a lung transplant on the basis of persistent NTM infection.

Our results differ from a 2003 multicenter US survey (*9*) in 2 crucial ways. First, the overall prevalence of NTM of 4.2% during the study period in the United Kingdom was much lower than the 13% reported for the study done in the United States. This could be caused in part by lower sampling frequency, occurring annually in most UK clinics, compared with 3 times annually as reported in the US study. The proportion of patients having >2 positive cultures was similar: 3.9% for the US study and 3.0% for our study. Furthermore, the denominator used for prevalence estimation may have been overestimated in this study: it may have included some patients missed for annual culture; and particularly for pediatric clinics, the denominator would include non–sputum producers (cough swabs are normally not cultured for NTM). The questionnaire we used did not specify which source documents respondents used; therefore, recall bias may be an issue. However, all UK centers extract and report NTM data to a national database annually.

The second striking difference between this study and the US study was that rapid-growing mycobacteria (especially MABSC) predominated in this survey while MAC predominated in the US study. Our results are more in keeping with subsequent European and Middle East prevalence studies (*7,10,12*). Rapid-growing mycobacteria such as MABSC are now recognized to be of greater clinical importance in CF than MAC (*1*), and a recent US single-center survey (*13*) has shown M. abscessus to be the current dominant NTM and to be associated with evidence of clinical decline.

Figure. Regional variation in percentage of cystic fibrosis patients in whom nontuberculous mycobacteria were isolated, United Kingdom, 2009. Percentages represent combined clinics within participating regions. Nineteen of 23 adult and 24 of 29 pediatric CF centers, accounting for 7,122 of 8,513 (84%) CF patients, participated in the survey.

As in previous studies, we found marked geographic variation in prevalence: Olivier et al. found higher rates in coastal centers in the United States (*9*), and Roux et al. found the highest prevalence in west and southwest France (*10*). The higher prevalence in the south of the United Kingdom could be caused by different sampling rates, but we found no systematic difference in screening policy between different UK regions. Climatic differences are relatively small across a small temperate island such as the United Kingdom. Geology could be another factor; NTM acquisition has been linked with water from aquifers (*12,14*) which are present mainly in the younger rocks in southeastern United Kingdom.

The varied threshold for instigating NTM treatment is perhaps not surprising in view of the lack of evidence to guide this. Although there is no clear evidence to justify treatment on positive cultures alone, some centers may have lowered their threshold in the hope that (by analogy with *Pseudomonas aeruginosa*) aggressive early treatment might lead to eradication. Refusal of lung transplantation is clearly an issue and appears to affect the threshold for treatment, though the rationale for refusal by transplant centers remains controversial (*15*).

Conclusions

This survey highlights the growing importance of NTM as a CF pathogen, the importance of routine, standardized surveillance, and the need to find out how best to manage NTM in CF. This will require further research into environmental, microbial, and host factors influencing acquisition and disease progression of NTM in the CF population.

Acknowledgments

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Dr Seddon is the Consultant Respiratory Paediatrician at the Royal Alexandra Children's Hospital, Brighton, UK, and Chair of the Respiratory and Cystic Fibrosis Clinical Studies Group of the UK Medicines for Children Research Network.

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Reducing Visceral Leishmaniasis by Insecticide Impregnation of Bed-Nets, Bangladesh

Dinesh Mondal, M. Mamun Huda, Mithun Kumar Karmoker, Debashis Ghosh, Greg Matlashewski, Shah Golam Nabi, and Axel Kroeger

The effect of insecticide-treated materials on reducing visceral leishmaniasis (VL) is disputable. In Bangladesh, we evaluated the effect of a community-based intervention with insecticide impregnation of existing bed-nets in reducing VL incidence. This intervention reduced VL by 66.5%. Widespread bed-net impregnation with slow-release insecticide may control VL in Bangladesh.

The governments of Bangladesh, India, and Nepal have committed to eliminate visceral leishmaniasis (VL) by 2015 (*1*). Reducing VL incidence by controlling sandflies, the vector of *Leishmania* spp. parasites, through integrated vector management is a key strategy of elimination programs (*2*). Community-based intervention with insecticide-treated materials, such as distribution of long-lasting insecticide–treated bed-nets or mass bed-net impregnation programs with slow-release insecticide tablets, could be possible vector-control components of integrated vector management if they are found effective in reducing VL incidence (*3*). We evaluated the effect of a community-based intervention with impregnation of existing bed-nets in reducing VL incidence in VL-endemic villages of subdistrict (upazila) Godagari, district Rajshahi, Bangladesh.

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The Study

The study comprised all 72 VL-endemic villages in Godagrai, distributed in 5 unions (Deopara, 36; Rishikul, 15; Gogram, 12; Pakuria, 6; and Mohanpur, 3). The intervention area was 36 villages in Deopara union comprising 2,512 households (11,426 inhabitants), and the control area was the 36 villages from other 4 unions comprising 3,143 households (14,021 inhabitants) (Figure 1). The bed-net impregnation intervention program with KO Tab 1-2-3 (Bayer Environmental Science, Bayer [Ply] Ltd., reg. no. 1968/011192/07, 21 Isando, South Africa, CODE 05682036 C) was conducted during February–March 2008. All households from all 79 villages in Deopara union, including households in 36 VL-endemic villages, were invited to participate in bed-net dipping (Figure 1). Details about the surveys and intervention are given in the online Technical Appendix (wwwnc.cdc.gov/EID/article/19/7/12-0932-Techapp1.pdf). We measured VL incidence in the intervention and control areas before and after intervention during September 2006–March 2007 and December 2009–January 2010, respectively. Household screening for VL cases in the previous 12 months was performed by trained field research assistants. Past VL cases were confirmed through document analysis and checking of hospital registers. A new VL case was defined by using the definition for new VL case of the National Kala-azar Elimination Guideline (*4*). VL incidence was expressed by number of VL cases (newly found plus past VL cases) per 10,000 persons. The field research assistants also conducted an in-depth interview with each household head by using a structured questionnaire in every 11th household and in households where they found new and past VL cases to collect sociodemographic characteristics of the surveyed community and VL-related knowledge and practice. A total of 556 household heads (254 and 302, respectively, in the intervention and control areas) were interviewed. Sociodemographic and knowledge, attitude, and practice variables between 2 areas with p values <0.2 were extended to 5,655 households by using statistical tools, and the validity was checked by comparing the distribution of each variable before and after random extension (online Technical Appendix Table). This helped us to investigate the eventual confounding effect of socioeconomic and knowledge, attitude, and practice variables on VL incidence reduction.

We evaluated the effect of the intervention on VL incidence in different ways. First, we compared reduction of VL incidence at the population level. Second, we compared reduction of VL-affected households in the 2 areas by a difference-in-difference method. Then, we examined the consistency of the effect of the intervention by measuring protection of the population from VL in the intervention area and protection of households from

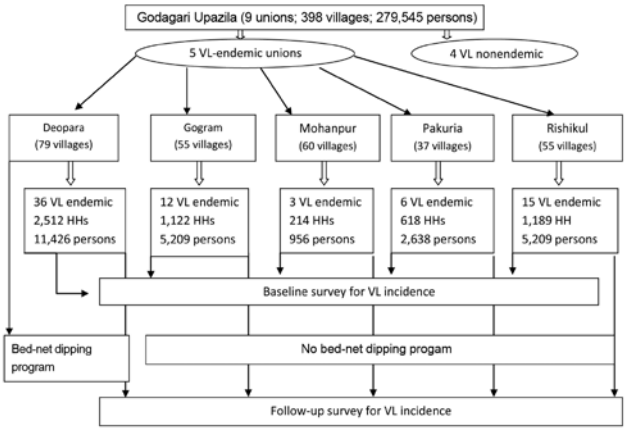

Figure 1. Design of study of reducing visceral leishmaniasis by insecticide impregnation of existing bed-nets, Bangladesh, 2006–2010. VL, visceral leishmaniasis; HH, households.

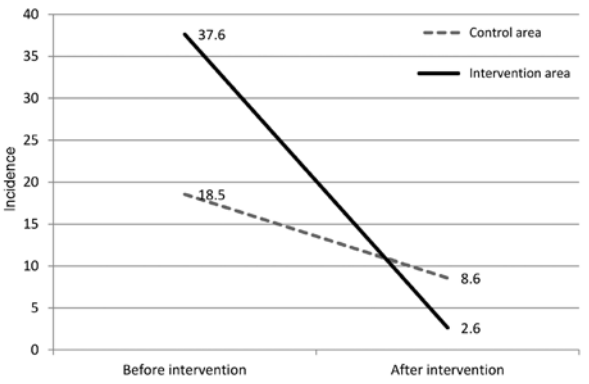

Figure 2. Visceral leishmaniasis incidence (cases per 10,000 persons) in intervention and control areas before and after intervention, Bangladesh, 2006–2010.

VL by the intervention through unadjusted and adjusted longitudinal logistic regression models. Data management and statistical analysis were conducted by using Epi Info version 3.2.2 (Centers for Disease Control and Prevention, Atlanta, GA, USA) and Stata 10.1 (Stata Corp, College Station, TX, USA), respectively. The International Centre for Diarrhoeal Disease Research, Bangladesh, and the Ethical Review Committees of the Special Program for Research and Training in Tropical Diseases/World Health Organization (WHO) approved the study. Informed written consent was obtained from each household head and from the persons with suspected VL for any study-related interventions.

The 2 areas differed regarding knowledge of the household head about VL symptoms, VL transmission, and household education (online Technical Appendix). A total of 2,239 (89.1%) of the 2,512 household heads from the study area of Deopara participated in the bed-net dipping. The use of impregnated bed-nets was also very high (99.8%), as found by random nightly observation in a subsample of households in the intervention area.

Before intervention, 69 VL cases were found, resulting in a VL incidence of 27 per 10,000 persons in the study area. VL incidence in the intervention area, 37.6 cases per 10,000 persons (43/11,426), was significantly higher than in the control area (18.5/10,000) (26/14,021; p = 0.0036). In intervention and control areas, 3 and 4 households, respectively, had multiple persons with VL. After intervention, VL incidence in intervention and control areas was 2.6 (3/11,426) and 8.6 (12/14,021) cases per 10,000 persons, respectively. During follow up, annual VL incidence declined in both areas, but the reduction was significantly greater in the intervention area (decrease of 35 cases/10,000 persons) than in the control area (decrease of 9.99/10,000; p = 0.001) (Table 1; Figure 2). The effect of community-level intervention, measured by difference-in-difference method, was 66.5% (Table 1). Using odds ratios in the longitudinal logistic regression model, we

Table 1. VL incidence and affected households before and after bed-net impregnation program, Bangladesh, 2006–2010*

Group	Bed-net impregnation		Rate changes (p value)	% Reduction† compared with control (p value)‡
	Before, no. (%) affected	After, no. (%) affected		
HH§				
Intervention, n = 2,512	40 (15.92)	3 (1.19)	–14.73 (<0.0001)	–70.52% (0.0007)
Control, n = 3,143	21 (6.68)	10 (3.18)	–3.50 (0.0476)	
Total, n = 5,655	61 (10.79)	13 (2.30)	–8.49 (<0.0001)	
Population¶				
Intervention, n = 11,426	43 (37.63)	3 (2.63)	–35.01(<0.0001)	–66.49% (0.001)
Control, n = 14,021	26 (18.54)	12 (8.56)	–9.99 (0.023)	
Total, n = 25,447	69 (27.12)	15 (5.89)	–21.22 (<0.0001)	

*VL, visceral leishmaniasis; HH, households.
†Effect of intervention: (B/A) – (D/C) Where A = baseline value for VL-affected HH per 1,000 HH/VL incidence per 10,000 persons in the intervention area; B = post-intervention value for VL-affected HH per 1,000 HH/VL incidence per 10,000 persons in the intervention area; C = baseline value for VL-affected HH per 1,000 HH/VL incidence per 10,000 persons in the control area; D = post-intervention value for VL-affected HH per 1,000 HHs/VL incidence per 10,000 persons in the control area. The effect is negative or positive if the VL-affected HH per 1,000 HHs/VL incidence per 10,000 persons is decreased or increased after intervention. Then the percentage reduction by intervention is calculated as (EI/[A]) × 100.
‡p values were calculated by Z statistic for pre- or post-rate differences between intervention and control areas.
§Incidence per 1,000 HH.
¶Incidence per 10,000 persons.

Table 2. Estimation of protection of households by the VL intervention using longitudinal logistic regression model with and without adjustment for confounders, Bangaladesh, 2006–2010*

Model; parameter	Odds ratio (95% CI)	Estimated protection by intervention at household level, % (95% CI)	p value
Simple, without adjustment for confounders; intervention	0.13 (0.030–0.557)	87 (44.3–97.0)	0.006
Full model, with adjustments for confounders			
Intervention	0.13 (0.03–0.56)	87 (44.3–97.0)	0.006
Family size >5 persons	1.75 (0.99–3.11)		0.054
HH head occupation, labor	2.38 (1.37–4.12)		0.002
Precarious (mud/thatched) house	4.64 (0.56–38.69)		0.156
HH head without any knowledge of VL symptom	0.25 (0.13–0.46)		<0.001
HH head without any knowledge of VL transmission	0.57 (0.33–0.98)		0.042
Having bed-net at home	0.49 (0.12–1.98)		0.319
Use of bed-net for protection against mosquito bites	2.57 (0.81–8.21)		0.109

*The intervention effect and covariates are tested in 2 different panel logistic regression models; simple not controlling for any covariates, full model controlling for confounders. VL, visceral leishmaniasis; HH, household.

found that 85.8% (95% CI 44.0%–96.5%; p = 0.005) of the population in the intervention area was protected from VL by the intervention.

The total number of household heads was 5,655, with 2,512 and 3,143 in the intervention and control areas, respectively. Before intervention, VL-affected households were 15.9 and 6.7 per 1,000 households in the intervention and control areas, respectively. After intervention, VL-affected households declined 13 times and 2 times, respectively, in the intervention and control areas compared with VL-affected households before intervention. The effect of the intervention in reducing VL-affected households in the intervention area compared with the control area was 70.5% by difference-in-difference analysis (Table 1). Again, using odds ratios in the longitudinal logistic regression model, we estimated the crude protection of households in the intervention area from VL by the intervention as 87% compared with those in the control areas. The protective effect of the intervention remained independent when adjusted for possible confounders (Table 2).

Conclusions

The community-based bed-net impregnation with slow-release insecticide significantly reduced VL incidence in VL-endemic areas. We used the difference-in-difference method for impact calculations because it is recommended by impact evaluation experts when effects of disease significantly differ between intervention and control, such as in our study (*5–10*). The protective effect was consistent and independent, as shown by the longitudinal logistic regression model. The differences in calculated effect and estimated protection at the household and community levels were due to households with multiple VL cases. Our findings agree with those of Ritmeijer et al. (*11*), who found a 59% reduction in VL by bed-net impregnation in Sudan. Our findings, however, were not consistent with those of Picardo et al. (*12*), who found no additional protection by random villagewise distribution of commercial insecticide–treated bed-nets compared with existing vector-control practice in India and Nepal. This discrepancy might be explained by the different delivery (commercial bed-net vs. existing bed-net impregnation) and coverage achieved (patchy villagewise vs. all villages in the area) by the intervention. We recommend mass coverage of VL-endemic villages with bed-net impregnation with slow-release insecticide for controlling VL in Bangladesh.

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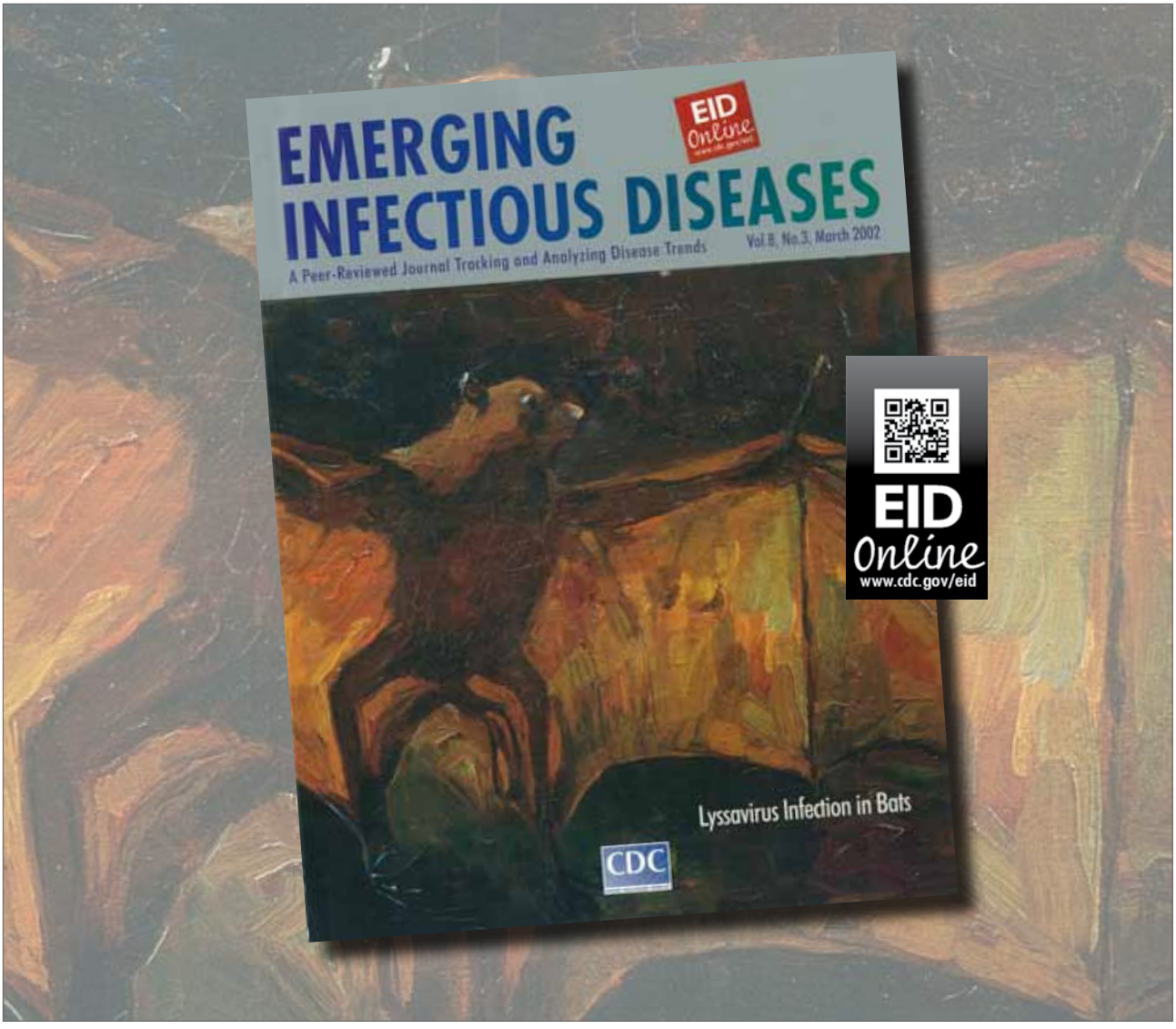

Genetic Variants of *Orientia tsutsugamushi* in Domestic Rodents, Northern China

Meng Zhang, Zhong-Tang Zhao, Xian-Jun Wang, Zhong Li, Lei Ding, Shu-Jun Ding, and Hui-Li Yang

We screened *Orientia tsutsugamushi* from 385 domestic rodents and 19 humans with scrub typhus in rural Tai'an District, Shandong Province, a new scrub typhus epidemic area in northern China. Sequence analysis identified 7 genotypes in the rodents, of which 2 were also identified in the humans.

Orientia tsutsugamushi, the causative agent of scrub typhus, is widely prevalent in the Asia–Pacific region and causes an estimated 1 million cases per year (*1*). It is characterized by dramatic genetic diversity (*2*). In the 1960s, complement fixation initially identified *O. tsutsugamushi* as Karp, Gilliam, and Kato types (*3*), which are widely used as tested antigens in serologic assays. Since then, >20 antigenic variants of *O. tsutsugamushi* have been identified by immunologic and molecular methods (*2*). The 56-kDa type-specific antigen (TSA), which is one of the major immunogens of the agent and is associated with pathogenesis, has been commonly used for type designation (*4–6*).

Scrub typhus is a traditional tropical rickettsiosis. However, since 1986, it has emerged and spread rapidly in temperate zones; the epidemic season is mainly in autumn and winter (*7*,*8*). In the late 1990s, the district of Tai'an, west of Shandong Province, northern China (116°20′–117°59′E, 35°38′–36°28′N), became a new epidemic area of autumn–winter type scrub typhus (*9*); however, few epidemiologic and molecular investigations of the transmission cycle of the disease have been conducted in this area. We therefore investigated the prevalence of *O. tsutsugamushi* infection among rural domestic rodents in the newly developed epidemic area, evaluated the genotypic diversity according to the variations in the 56-kDa TSA gene, and explored the genetic relationship between strains circulating among domestic rodents and those infecting humans.

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The Study

From September 2010 through March 2012, rodents were trapped in rural residences from 2 county-level divisions (Xintai and Daiyue) in Tai'an. After each rodent was numbered and species identified, the spleen was dissected for DNA extraction.

During 2010–2011, scrub typhus patients from Xintai and Daiyue were recruited from 7 hospitals in Tai'an. Before the patients received antimicrobial drugs, whole blood samples were collected and anticoagulated with EDTA-Na_2. Eschar specimens, if available, were collected after spontaneous desquamation.

DNA was extracted from each specimen by using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Considering the higher sensitivity and specificity, seminested PCR was used to amplify the coding sequences spanning variable domain I–VD III of the 56-kDa TSA with previously described primers A (5′-TTTCGAACGTGTCTTTAAGC-3′), B (5′-ACAGATGCACTATTAGGCAA-3′), and E (5′-GTTGGAGGAATGATTACTGG-3′) (*10*), which yielded fragments of ≈733 nt. To avoid contamination, PCR preparation was performed in an area separate from DNA extraction, and negative controls were included in each step. Nucleotide sequences of *O. tsutsugamushi* determined in this study were deposited in GenBank under accession nos. JX202566–JX202568, JX202573, JX202578, JX202579, and JX202580–JX202589. Theses sequences were aligned with 38 reference sequences of the *O. tsutsugamushi* 56k-Da TSA gene retrieved from GenBank by using MEGA software with the ClustalW algorithm (*11*). The phylogenetic tree was constructed by using MEGA software with the minimum-evolution method and the Kimura 2-parameter model, with bootstrapping for 1,000 replications. A percentage nucleotide identity matrix was calculated by using MegAlign (Lasergene; DNASTAR Inc., Madison, WI, USA).

The species of the 385 captured rodents were 244 *Rattus norvegicus*, 139 *Mus musculus*, and 2 *R. rattus*. *O. tsutsugamushi* DNA was detected by PCR in 10 (2.6%) rodents captured in October, November, December, and March; 6 (4.3%) rodents were *M. musculus* and 4 (1.6%) were *R. norvegicus*. Rates of *O. tsutsugamushi* positivity in domestic rodents captured in spring, summer, autumn, and winter were 5.3%, 0, 3.1%, and 1.2%, respectively (Table). No significant difference was observed in the rate of *O. tsutsugamushi* positivity determined by PCR among the 3 species of rodents ($p = 0.278$) or among seasons ($p = 0.274$).

Among the 10 sequences obtained from rodents, we identified 3 genogroups (Kawasaki-related, Fuji-related, and novel genogroup) involving 7 genotypes with ≥1 nt difference. The genotypes showed 69.7%–99.9% identity

Table. Rates of *Orientia tsutsugamushi* positivity among domestic rodents , Tai'an, China, September 2010–March 2012*

Rodent species	Season, no. positive/no. tested (%)				
	Spring	Summer	Autumn	Winter	Total
Mus musculus	2/18 (11.1)	0/21 (0)	3/60 (5.0)	1/40 (2.5)	6/139 (4.3)
Rattus norvegicus	1/39 (2.6)	0/31 (0)	3/131 (2.3)	0/43 (0)	4/244 (1.6)
R. rattus	0	0	0	0/2 (0)	0/2 (0)
Total	3/57 (5.3)	0/52 (0)	6/191 (3.1)	1/85 (1.2)	10/385 (2.6)

*Determined by PCR.

with each other. *M. musculus* and *R. norvegicus* rodents in Tai'an could serve as reservoirs of 5 and 3 genotypes, respectively. Genotypes KWS1–4 clustered with the Kawasaki genotype (96%–96.5% identity). Genotype FJS was closely related to the Fuji genotype (96.5% identity), which was originally isolated from *Leptotrombidium fuji* mites in Japan (*12*). Genotypes SDM1 and SDM2 clustered in an independent clade, which had 70.4%–77.9% identity with reference strains (Figure).

Whole blood or eschar specimens were obtained from 19 human patients, among whom 6 (31.6%) were infected with *O. tsutsugamushi* according to seminested PCR. Two genotypes (KWS1 and KWS2), which were identified in domestic rodents and belonged to Kawasaki-related genogroups, were also found in the patients (Figure).

Conclusions

Our study demonstrated the prevalence of 7 genotypes of *O. tsutsugamushi* in the new epidemic area in Tai'an, northern China. Genetic diversity of *O. tsutsugamushi* was observed within rodent species. Given that KWS1 and KWS2 identified in domestic rodents could cause human infection, prevalence of *O. tsutsugamushi* infection in domestic rodents might be a public health concern. Natural

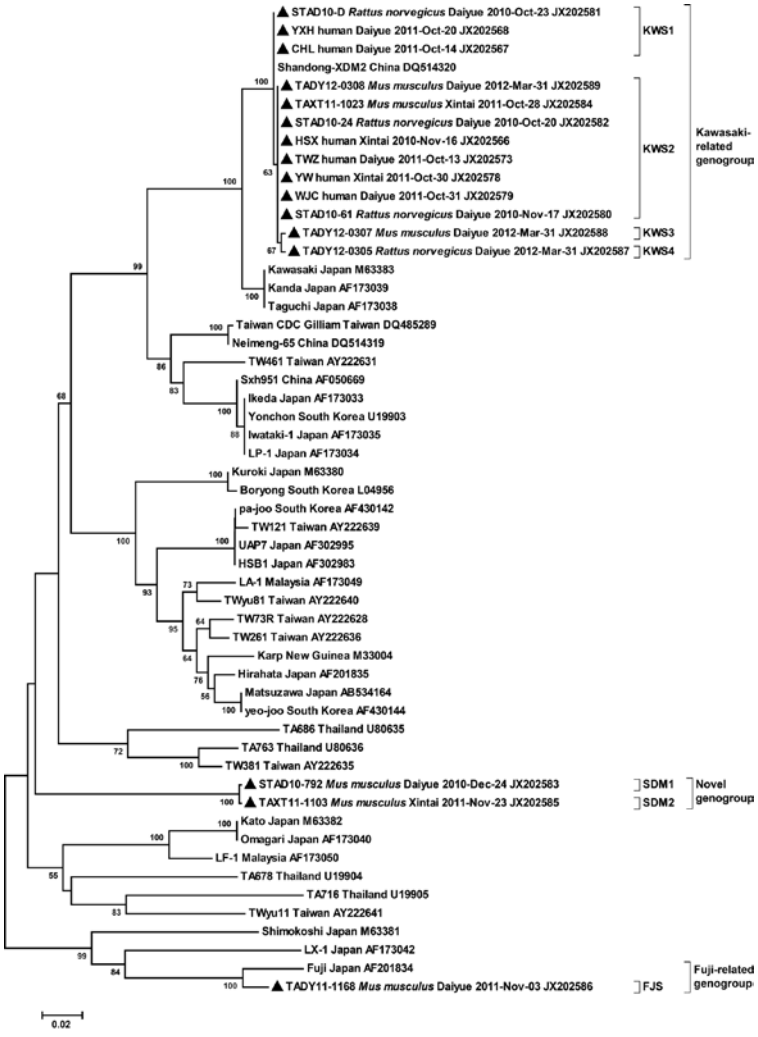

Figure. Phylogenetic relationships of *Orientia tsutsugamushi* detected in domestic rodents and human patients with scrub typhus in rural areas of Tai'an, Shandong Province, China, September 2010 through March 2012. Relationships were determined on the basis of the partial 56-kDa type-specific antigen gene of *O. tsutsugamushi* by the minimum-evolution method with the Kimura 2-parameter distance model. Bootstrap values ≥50% are shown at the branches. Location and GenBank accession numbers are indicated for each sequence. Sampling sources and sampling date are also indicated for sequences determined in the study. Solid triangle indicates sequences determined in this study. Scale bar indicates genetic distance.

infection with the KWS2 genotype of *O. tsutsugamushi* in rodents was found during March in Tai'an. Periodic molecular surveillance of *O. tsutsugamushi* in local areas might help predict changes in the epidemic features of scrub typhus. Physicians should not arbitrarily exclude a diagnosis of scrub typhus for patients with fever of unknown origin during nonepidemic seasons.

The Kawasaki and Fuji strains of *O. tsutsugamushi* were considered to be of low virulence (*12*,*13*). Cross-reactivities of the Kawasaki strain with 1–18 anti-Gilliam monoclonal antibodies and of the Fuji strain with 5D-3 anti-Kato monoclonal antibodies were observed (*12*). The 56-kDa TSA is responsible for adhesion to and invasion of the host cells, and its variable domains might serve as epitopes (*5*,*14*). Although the identity of the KWS1–4 and FJS genotypes and their prototypes was >96%, changes in virulence and antigenicity might occur because of minor variation. Among the scrub typhus patients from Xintai and Daiyue, Kawasaki-related genotypes were predominant. Antigenic types of these genotypes remain to be determined. Those that are antigenically distinguished from the commonly used antigens should be included in the antigen panel of serologic assays in the local area, which would prompt the diagnosis and prevention of scrub typhus for local residents and travelers.

Identity of SDM1 and SDM2 with the reference strains was ≤77.9%, and they formed a distinct cluster, indicating that they might represent novel genotypes. Infection of scrub typhus patients with SDM1, SDM2, and Fuji-related genotypes has not been reported. Investigation of host ranges, invasiveness, and virulence of newly defined genotypes of *O. tsutsugamushi* in local areas will help determine their pathogenic potential for humans.

Expansion of genetic diversity of *O. tsutsugamushi* was expected to occur in maintenance hosts (*15*). We are currently screening *O. tsutsugamushi* in chigger mites collected from the captured rodents to evaluate the role of domestic rodents in the transmission of scrub typhus to humans. Assiduous surveillance of genetic variations of *O. tsutsugamushi* in hosts and identification of their pathogenic potentials is essential for the improvement of diagnostic capacity, vaccine development, and assessment of epidemiologic role.

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Undetected Multidrug-Resistant Tuberculosis Amplified by First-line Therapy in Mixed Infection

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Infections with >1 *Mycobacterium tuberculosis* strain(s) are underrecognized. We show, in vitro and in vivo, how first-line treatment conferred a competitive growth advantage to amplify a multidrug-resistant *M. tuberculosis* strain in a patient with mixed infection. Diagnostic techniques that identify mixed tubercle bacilli populations are needed to curb the spread of multidrug resistance.

As the number of multidrug-resistant tuberculosis (TB) cases continues to rise, so does the amplification of multidrug-resistant *Mycobacterium tuberculosis* strains during treatment (*1*,*2*). This amplification is generally assumed to result from in vivo evolution of drug resistance caused by poor therapy compliance or, in high-incidence settings, from exogenous reinfection with a multidrug-resistant strain. We report a case in which emergence of multidrug resistance did not result from in vivo acquisition of drug resistance by a drug-sensitive strain or from exogenous reinfection with an already resistant strain. By integrating epidemiologic, microbiological, and molecular strain typing data with in vitro competitive growth experiments, we provide evidence for an initial mixed infection with a drug-sensitive strain and an undetected drug-resistant strain that outgrew the sensitive strain under the selection pressure of first-line chemotherapy.

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M. tuberculosis strains in sputum from TB-infected patients or in samples from the disease site are generally identified by strain typing a single broth culture or colony grown on solid medium. However, this method does not enable identification of mixed infections, and any treatment regimen would be determined on the basis of the drug sensitivity of the strain with the fastest growth rate in the in vitro culture. Use of suboptimal drug combinations could lead to selection of a slower growing, drug-resistant strain already present in the host and thus to treatment failure.

Studies of artificially mixed *M. tuberculosis* strains before and after culture showed that culturing can reduce the clonal complexity of the strains and that, in most samples (6/10), only 1 strain will be identified in mixed infections after culture (*3*). This suggests that mixed infections and clonal complexity are underrepresented in culture-based diagnoses of TB. In support of this suggestion, the results of molecular-based methods that use strain-specific PCR showed that 2.1%–19.0% of patients with active TB in moderate to high incidence countries were simultaneously infected with ≥2 strains (*1*,*2*,*4*–*10*).

Possible co-infection of patients with drug-sensitive and drug-resistant *M. tuberculosis* strains has been described (*1*,*2*), and modeling of the effect of such co-infection on the long-term dynamics of tuberculous infection has led to the hypothesis that persons with this type of infection may retain small populations of drug-resistant bacteria that can flourish after the host receives treatment (*11*). van Rie et al. showed the amplification of a drug-resistant strain after treatment and postulated selection of drug-resistant strains from an initial mixed infection through antimicrobial drug pressure (*2*). We confirm this hypothesis by combining detailed longitudinal clinical and microbiological observation with the use of novel in vitro growth competition assays to study 2 co-infecting patient strains in the presence and absence of the primary drug used in treatment.

The Study

The 2 *M. tuberculosis* strains were isolated from a 68-year-old man from Portugal. He did not have HIV and was treated as a confined inpatient, limiting the possibility that this was not a true in vivo mixed infection. Using a novel approach, we correlated in vitro growth and treatment characteristics for the patient strains with the in vivo strain predominance and persistence of a less-fit, drug-resistant strain. All samples were obtained with approval from St. Mary Hospital's (London, UK) Research Ethics Center (no. 07/H0712/85) and with the patient's written informed consent.

Details of the patient samples are in the Table. The initial bronchoalveolar lavage smear sample was positive

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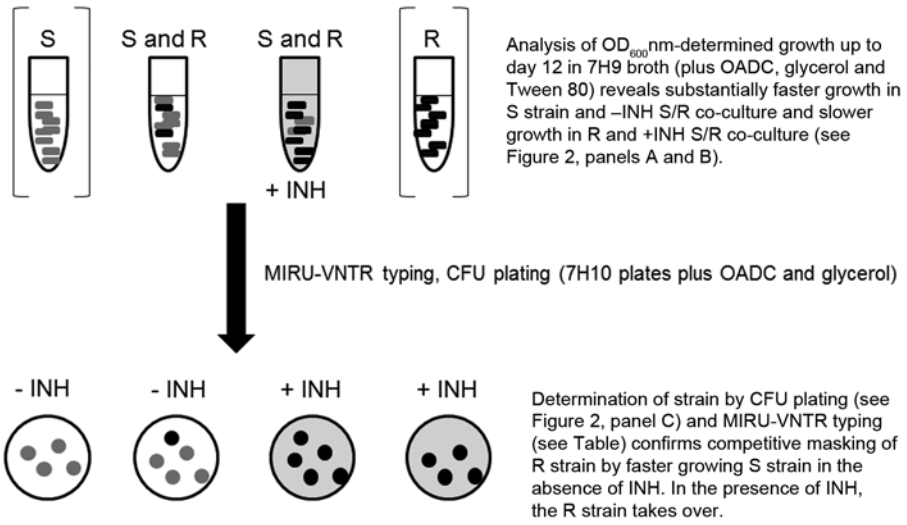

Figure 1. *Mycobacterium tuberculosis* co-culture competition experiment in a study of the amplification of multidrug resistance induced by first-line treatment of a mixed *M. tuberculosis* infection. The results suggest competitive advantages in vitro, which may account for patient strain phenotype in vivo. S, drug sensitive; R, drug resistant; OADC, oleic acid, albumin, dextrose, catalase growth supplement; OD_{600}, optical density read at 600 nm; -INH, without isoniazid; +INH, with INH; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat; CFU, colony-forming units.

for acid-fast bacilli (AFB); culture results were positive for fully sensitive *M. tuberculosis*. Treatment with isoniazid, rifampin, ethambutol, and pyrazinamide was begun. Because of the patient's alcohol use, his treatment was managed on an inpatient basis in a single-patient, negative-pressure room. Two months later, repeat sputum smears were positive for AFB, and culture results were positive for fully sensitive *M. tuberculosis*. After 4 months of treatment, the patient's clinical signs had not improved, and his sputum smear was still positive for AFB. Culture results for the sputum sample were positive for *M. tuberculosis* resistant to isoniazid and ethambutol; a modified treatment regime resolved the infection, and the patient was released the following month, by which time his smear and culture results were negative.

We molecularly characterized the strains by using mycobacterial interspersed repetitive unit–variable number tandem repeat (MIRU-VNTR) typing (*12*); results showed that the drug-sensitive and drug-resistant *M. tuberculosis* strains were 2 distinct strains (Table) rather than 1 sensitive strain that had become resistant through mutagenesis. Because the patient was isolated while an inpatient, exogenous

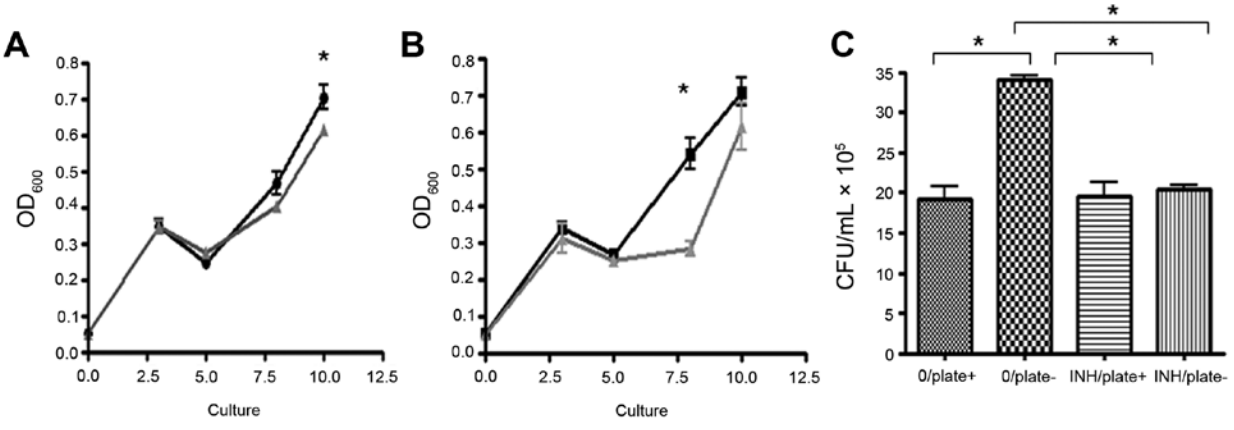

Figure 2. Analyses of the amplification of multidrug-resistant *Mycobacterium tuberculosis* during treatment of a drug-sensitive (S) strain in a mixed infection (i.e., infection with drug-resistant [R] and S strains). In the presence of isoniazid (INH), the faster growing S strain lost its competitive advantage, and the R strain became more prevalent. A–C) Data are means of 3 independent replicates with SE bars. A) Single strain growth analyses of S (black circles) and R (gray triangles) *M. tuberculosis* strains. Growth was measured by optical density at 600 nm (OD_{600}). *p<0.05. B) Competitive growth analyses of mixed strains alone (black squares) and with 0.2 μg/mL INH (gray triangles). Growth, in triplicate, in 7H9 broth plus OADC (oleic acid, albumin, dextrose, and catalase growth supplement), glycerol, and Tween 80 was measured by optical density at OD_{600}. Statistical analyses were performed on triplicate samples by using 2-way analysis of variance. *p<0.05. C) Identification of the predominant strain in mixed cultures with and without 0.2 μg/mL INH (INH/plate+ and INH/plate-, respectively). Strains were identified on day 7 by plating a 10-fold dilution series of co-cultures onto 7H10 agar, plus OADC and glycerol, with or without 0.2 μg/mL INH (0/plate+ and 0/plate-, respectively) and incubating for 2 weeks at 37°C. Statistical analyses were conducted on triplicate samples by using a 2-tailed *t*-test. *p<0.02. CFU, colony-forming units.

Table. Details for samples used in a study of the amplification of multidrug-resistant tuberculosis, resulting from competitive growth advantage, during treatment of a drug-sensitive *Mycobacterium tuberculosis* strain in a mixed infection*

Sample	Isolate	Smear	Resistance	MIRU-VNTR
Bronchoalveolar lavage, obtained February 2008	S	+	None	3243323125153242244235-1
Sputum, obtained April 2008	S	++	None	3243323125153242244235-1
Sputum, obtained June 2008	R	+++	INH, ETB	-2434233251533445-443330
Co-cultures 1) broth culture + INH, 2) colonies from broth culture – INH on plate with INH, 3) colonies from broth culture + INH on plates – INH and 4) colonies from broth culture + INH on plates – INH	R	NA	INH	-2434233251533445-443330
Co-cultures 5) broth culture – INH and 6) colonies from broth culture – INH on plates – INH	S	NA	NA	3243323125153242244235-1

*MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat; S, drug sensitive; +, ++, and +++, relative burden of acid-fast bacilli; R, drug resistant; INH, isoniazid; ETB, ethambutol; NA, not applicable.

reinfection with a primary drug-resistant strain was ruled out. In addition, treatment compliance was directly observed, so in vivo development of drug resistance caused by poor compliance was also ruled out. Thus, it is highly likely that the patient was harboring a mixed infection of drug-sensitive and drug-resistant strains when he initially sought care at the clinic. Such a co-infection would not have been detected because single-colony or broth cultures are commonly used for strain typing, and these techniques would give the fastest growing strain a competitive advantage. Thus, we devised a competitive growth assay to determine if the patient had a mixed infection and to provide correlating in vitro and in vivo evidence of mixed infection (Figure 1).

For the in vitro growth analyses of the 2 strains, we inoculated broth cultures and measured growth at an optical density of 600 nm, characterized the dominant strain by using MIRU-VNTR, and quantified colony-forming units on agar plates in the presence and absence of isoniazid. At several points during logarithmic growth, the drug-sensitive strain grew substantially faster than the resistant strain (Figure 2, panel A), suggesting that without the selective pressure of isoniazid, the sensitive strain would be most prevalent in a mixed infection.

For the in vitro competition assays, the strains were mixed (1:1), and isoniazid (0.2 μg/mL) was or was not added before measurement of growth and determination of the dominant strain. In the presence of isoniazid, the growth rate was lower, suggesting that the drug-resistant strain outcompeted the drug-sensitive strain to become the dominant strain (Figure 2, panel B). This was confirmed by MIRU-VNTR typing and growth analyses (Figure 2, panel C). These results indicate that 1) the drug-sensitive strain had a competitive growth advantage, causing this strain type to be identified as the sole infecting strain, and that 2) the drug-resistant strain gained the competitive advantage when isoniazid was added and became the predominant strain after treatment. These findings correlate precisely with the patient data (Table) and, we believe, is representative of the in vivo host infection.

Conclusions

We show the selection and subsequent clinically relevant emergence of a drug-resistant *M. tuberculosis* strain after treatment of a drug-sensitive strain in a patient with an initial mixed infection. This case illustrates the prospect of treatment failure for TB caused by mixed infection with strains with different drug susceptibility and growth rates. The proportion of cases of secondary multidrug resistance caused by such initial mixed infections is not known; however, the ability of the resistant strain to outcompete the sensitive strain under treatment and then to potentially transmit further may have substantial implications for the control and prevention of multidrug resistance.

The case also highlights the urgent need for improved diagnostic techniques that can routinely identify mixed populations of tubercle bacilli. Given the difficulty of detecting TB co-infections by using routine diagnostic microbiology techniques, co-infection is likely underrecognized. Co-infection can currently be ruled out only by using specialized techniques, such as molecular analysis of original sample (pre-culture); analysis of multiple colonies; or the GeneXpert assay (Cepheid, Sunnyvale, CA, USA) (*13*). Rapid detection of mixed infections with distinct drug susceptibility profiles would enable suitably tailored drug regimens from the start of treatment, which could prevent treatment failure and emergence and transmission of drug-resistant strains.

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The experiments were designed and conducted by S.M. H-W., and the paper was written by S.M. H-W, R.C. and A.L.

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Clinical Findings for Early Human Cases of Influenza A(H7N9) Virus Infection, Shanghai, China

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A novel strain of influenza A(H7N9) virus has emerged in China and is causing mild to severe clinical symptoms in infected humans. Some case-patients have died. To further knowledge of this virus, we report the characteristics and clinical histories of 4 early case-patients.

Avian influenza A(H7N9) virus normally circulates among birds; however, human infections with this virus were confirmed in China on March 31, 2013 (*1*,*2*). To help identify the best treatment strategies for influenza A(H7N9) virus infection, we summarized the clinical characteristics and outcomes for the first 4 patients who were transferred to Shanghai Public Health Clinical Center (SHPHCC) for treatment of influenza A(H7N9) virus infection. For each case, infection was confirmed by the Shanghai Municipal Centers for Disease Control and Prevention.

Case Reports

Clinical features of the 4 case-patients are listed in Table 1. All case-patients were 58- to 73-year-old married men, farmers or retirees, and long-term residents of Shanghai (Fengxian, Baoshan, Songjiang, and Pudong districts, respectively). Case-patient 1 had a history of coronary heart disease and hepatic schistosomiasis; case-patient 2 had no history of chronic disease; case-patient 3 had a history of hypertension and gout; and case-patient 4 had a history of hypertension and repetitive cough for >10 years during spring and autumn.

Case-patient 1 raised chickens at home. Case-patients 2–4 had no clear history of close contact with poultry; however, each had visited various farmers' markets that sold live poultry. None of the patients raised pigeons or live in or near a heavily pigeon-infested area.

Before being transferred to SHPHCC on April 6, 2013 (patients 1 and 2) and April 7, 2013 (patients 3 and 4), the 4 patients had been treated in local hospitals; infection with influenza A(H7N9) virus had been confirmed by real-time reverse transcription PCR of nasopharyngeal swab samples before transfer. The case-patients had cough and fever and had been expectorating sputum for ≈6–7 days before admittance to SHPHCC. In addition, all had experienced cold-like symptoms and fatigue before influenza-like symptoms developed. Case-patient 4 had cough and fever for 18 and 10 days, respectively, before being transferred to SHPHCC; his case was the most serious of the 4, and the disease progressed rapidly after he was transferred to SHPHCC.

Total leukocyte counts for case-patients 1–4 were within or slightly below reference values: 5.50, 5.95, 3.50, and 4.60 × 10^9/L, respectively (reference value 4.00–10.00 × 10^9/L). The proportions of neutrophils were normal or slightly high: 79.6%, 62.6%, 72.4%, and 68.0%, respectively (reference value 50.0%–70.0%). Laboratory test results at admission are shown in Table 2. Radiograph findings mainly included ground-glass opacity and consolidation (Figures 1, 2; online Technical Appendix Figures 1, 2, wwwnc.cdc.gov/EID/article/19/7/13-0612-Techapp1.pdf). Computed tomography (CT) scans and radiograph findings, along with clinical manifestations and laboratory test results, helped establish early diagnoses.

To ensure proper treatment/management of the patients, an emergency team was established; the team followed the procedures shown in online Technical Appendix Figure 3. All 4 case-patients were administered antimicrobial drugs and the antiviral drug oseltamivir. Case-patient 1 began treatment 6 days after the onset of hypoxia, when large areas of lung inflammation were seen on radiographs. Case-patient 2 was treated 4 days after the onset of fever, when CT scan results revealed inflammation in the left upper lung lobe. Case-patient 3 began treatment 4 days after the onset of cough, sputum, and shortness of breath and after CT scan results revealed inflammation in the left lower lung lobe. Case-patient 4 began treatment 16 days after onset of high fever, dyspnea on exertion, and hypoxemia. Additional details for each patient are included below, and results of viral testing done at admission and 5 days later are shown in online Technical Appendix Table 1. Disease characteristics for infections caused by influenza virus

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subtypes H1N1, H5N1, and H7N9 are shown in online Technical Appendix Table 2.

Case-patient 1 was receiving noninvasive ventilator-assisted breathing when he arrived at SHPHCC. His oxygen saturation remained at ≈95%, and he was given continuous intravenous dopamine infusion. He had acute respiratory failure, coronary heart disease (stage 2 heart failure), and renal function insufficiency at admission. On April 11, 11 days after the onset of the symptoms and 2 hours after endotracheal intubation and mechanical ventilation began, he died from respiratory failure.

Case-patient 2 arrived at SHPHCC with a nasal cannula inserted to maintain oxygen saturation at 95%. His general condition improved steadily after commencing antiviral drug treatment, and he was discharged 18 days after illness onset.

Table 1.Clinical characteristics and treatment outcomes for 4 patients with early cases of influenza A(H7N9) virus infection, Shanghai, China*

Characteristic/treatment	Case-patient no. 1	2	3	4
Age, y/sex	73/M	65/M	67/M	58/M
Occupation	Farmer	Retiree	Retiree	Retiree
Location (district) in Shanghai	Fengxian	Baoshan	Songjiang	Pudong
Disease history	Coronary heart disease; chronic hepatic schistosomiasis	Hypertension; articular gout; benign prostatic hyperplasia	None	Hypertension
History of poultry exposure	At home	At live poultry markets	At live poultry markets	At live poultry markets
Date of last visit to live poultry market	NA	2013 Mar 29	2013 Mar 28	2013 Mar 19
Date of symptom onset	2013 Mar 31	2013 Apr 1	2013 March 30	2013 Mar 20
Date of infection confirmation	2013 Apr 6	2013 Apr 6	2013 Apr 7	2013 Apr 7
Date admitted to SHPHCC	2013 Apr 6	2013 Apr 6	2013 Apr 7	2013 Apr 7
Clinical symptoms present when admitted SHPHCC	6 d of fever (maximum temperature 39.3°C) and shortness of breath	6 d of fever (maximum temperature 39.3°C), and 2 d of cough	8 d of fever (maximum temperature 39.7°C) and cough	18 d of cough, 10 d of fever (maximum temperature 39.7°C), and 5 d with shortness of breath
Chest radiograph or CT findings	Bilateral GGO	Bilateral GGO	GGO in left lingular lobe and left inferior lobe	Extensive infiltrates, with pleural effusion, in lung (bilateral)
Antiviral drug treatment	Oseltamivir (150 mg/bid) on days 7–12 of illness	Oseltamivir (75 mg/bid) on days 4–17 of illness	Oseltamivir (75 mg/bid) on days 6–21 of illness	Oseltamivir (75 mg/bid) on days 16–23 of illness; oseltamivir (150 mg/bid) on days 17–32 of illness
Antibacterial drug treatment	Moxifloxacin on days 7–12 of illness	Ceftriaxone on days 4–5 of illness; moxifloxacin on days 6–17 of illness	Azithromycin on days 5–9 of illness; cefaclor on days 1–5 of illness; moxifloxacin on days 14–21 of illness	Moxifloxacin on days 18–21 of illness; piperacillin and tazobactam on days 18–21 of illness; meropenem on days 21–34 of illness; linezolid on days 25–32 of illness
Glucocorticoid treatment	Methylprednisolone (40 mg/d) on days 7–12 of illness	No	Methylprednisolone (40 mg/d) on days 5–12 of illness	Methylprednisolone (40 mg/bid) on days 16–37 of illness
Immunoglobulin treatment	Yes, on days 7–12 of illness	Yes, on days 6–12 of illness	Yes, on days 5–8 of illness	Yes, on days 16–37 of illness
ECMO treatment	No	No	No	On day 25 of illness
Oxygen use	Noninvasive ventilation on days 6–12 of illness	Oxygen inhalation through nasal tube on days 4–17 of illness	Oxygen inhalation through nasal tube on days 7-20 of illness	Noninvasive ventilation on days 17–19 of illness
Endotracheal intubation and mechanical ventilation	Yes, on day 12 of illness	No	No	Yes, on days 19–32 of illness
Status as of 2013 Apr 21	Died on day 12 of illness	Recovered, discharged on day 18 after illness onset	Recovered, discharged on day 21 after illness onset	Condition worsened, receiving invasive breath machine and ECMO treatment

*NA, not applicable; SHPHCC, Shanghai Public Health Clinical Center; CT, computed tomography scan; GGO, ground-glass opacity; bid, 2 times a day; ECMO, extracorporeal membrane oxygenation.

Table 2. Laboratory findings at admission for 4 patients with early cases of influenza A(H7N9) virus infection, Shanghai, China

Laboratory variable	Case-patient no. 1	2	3	4	Reference value
Leukocyte count, × 10^9/L	2.95	3.74	2.89	5.38	4.00–10.00
% Neutrophils	80.4	76.7	78.6	94.6	50.0%–70.0%
% Lymphocytes	13.5	18.2	15.4	2.4	20.0%–40.0%
Platelet count, × 10^9/L	71	82	172	75	85–303
Aspartate aminotransferase, U/L	86	77	45	172	8–40
Lactate dehydrogenase, U/L	886	492	209	906	109–245
Creatine phosphokinase, U/L	170	1,854	170	772	38.00–174
Creatine kinase isoenzyme MB, U/L	18	31	7	22	0–24

Case-patient 3 arrived at SHPHCC with a nasal cannula inserted to maintain oxygen saturation at 95%. He had a history of hypertension and gout. He was treated with oseltamivir, antimicrobial drugs, and steroids to suppress lung inflammation. His condition improved substantially, and he was discharged 21 days after illness onset.

Case-patient 4 arrived at SHPHCC in critical condition: oxygen saturation was 88%, and he had shortness of breath (30–35 breaths/min). He was immediately given noninvasive mechanical ventilation. One day after admission, his condition deteriorated; multiple organ dysfunctions in lung and kidney developed. His condition continued to deteriorate despite active treatment with oseltamivir and antimicrobial drugs. Severe hypoxemia developed. Two days after admission, invasive mechanical ventilation and then extracorporeal membrane oxygenation were implemented. The patient was still in critical condition on April 21, 2013.

Discussion

Clinical manifestations of disease in the 4 case-patients were consistent with those reported for other persons infected with influenza A(H7N9) virus (*3*). Case-patients 1 and 4 had a more severe disease course than case-patients 2 and 3. All patients sought medical care for unresolved fever, cough, expectoration of sputum, and shortness of breath. The severe cases progressed rapidly: body temperature was mostly sustained ≥39°C, and breathing was difficult and sometimes accompanied by hemoptysis. A rapid progression of acute respiratory distress syndrome

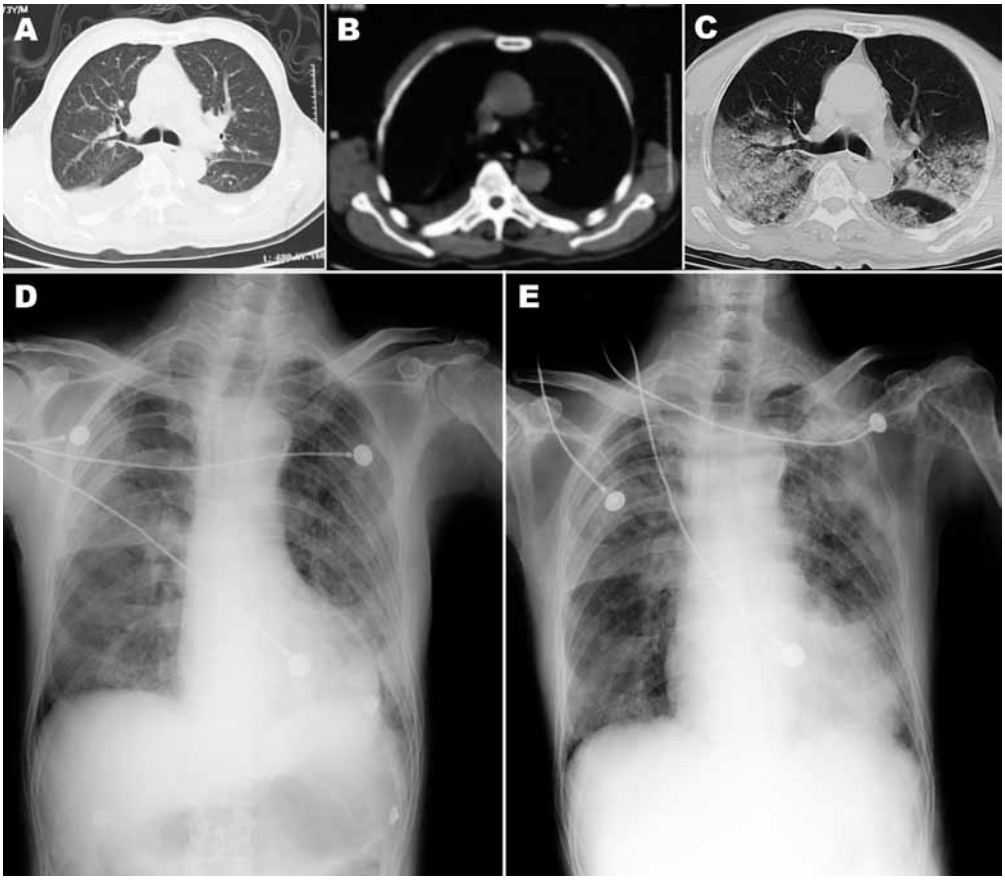

Figure 1. Chest computed tomography (CT) scan and radiograph images of patient (case-patient 1) in a study of 4 persons with early cases of influenza A(H7N9) virus infection, Shanghai, China. Images were taken 1, 5, 7, and 11 days after illness onset. A, B) CT scan images on day 1, showing bilateral pleural effusion but no obvious lesions. C) CT scan image on day 5, showing extensive ground-glass opacity and consolidation. D, E) x-ray images on days 7 and 11, respectively, showing reduced light transmittance on both sides of the lung.

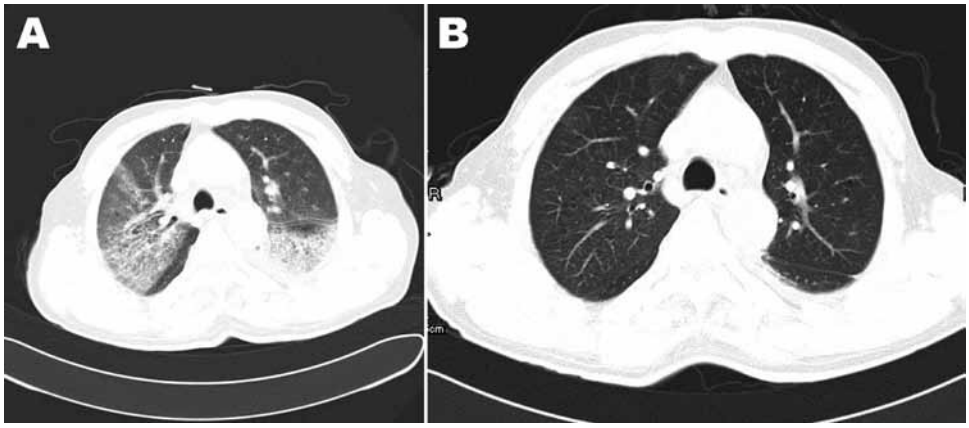

Figure 2. Chest computed tomography scan images of patient (case-patient 2) in a study of 4 persons with early cases of influenza A(H7N9) virus infection, Shanghai, China. A) Image taken 6 days after illness onset shows ground-glass opacity in the left lower and right upper lobes. B) Image taken 16 days after illness onset shows absorption of ground-glass opacity.

occurred in case-patients 1 and 4, along with mediastinal emphysema, shock, disturbed consciousness, and acute kidney injury. No close contacts of the 4 patients have had signs or symptoms of infection.

The currently available drug treatment for influenza A(H7N9) virus infection is neuraminidase inhibitors (e.g., oseltamivir). Their early use may be recommended (*10*) but is not always achieved. Case-patient 4 only began neuraminidase inhibitors 16 days after the onset of symptoms, by which time he was in a severe condition. Case-patient 1 was treated with oseltamivir 6 days after the onset of symptoms and, despite treatment, died 6 days after admission to SHPHCC. Earlier, higher doses combined with continuous treatment might improve patient outcomes (*5*). On the basis of clinical judgment, we now use 150 mg of oseltamivir twice daily for severe cases, monitoring for toxicity.

The benefits of oseltamivir treatment of influenza A(H7N9) virus infections are debatable; for example, case-patients 2 and 3 remained positive for the virus after 9–11 days of oseltamivir treatment (online Technical Appendix Table 1). Thus, it is essential to determine whether the virus has developed resistance to oseltamivir. Ineffectiveness of the oral oseltamivir formulations may also have contributed to treatment failure, especially for case-patients 1 and 4: the drug may not have been well absorbed, especially by patients in severe condition. If available in the future, systemic delivery of oseltamivir may be superior.

Of the 4 patients reported here, only case-patient 1 died shortly after admission to SHPHCC. He is also the only patient who had close contact with chickens. However, it is not clear that this contact contributed to the rapid progression of disease in case-patient 1, especially given the fact that case-patient 4, who is still in critical condition, also had rapid progression of disease. The other patients did not raise birds at home, but they visited live poultry markets.

Prompt and early communication of the clinical features of persons infected with avian influenza A(H7N9) virus is crucial to the development of effective treatment strategies (*6*). Research to understand the transmission pattern and effective control of this virus is urgently needed (*7–9*).

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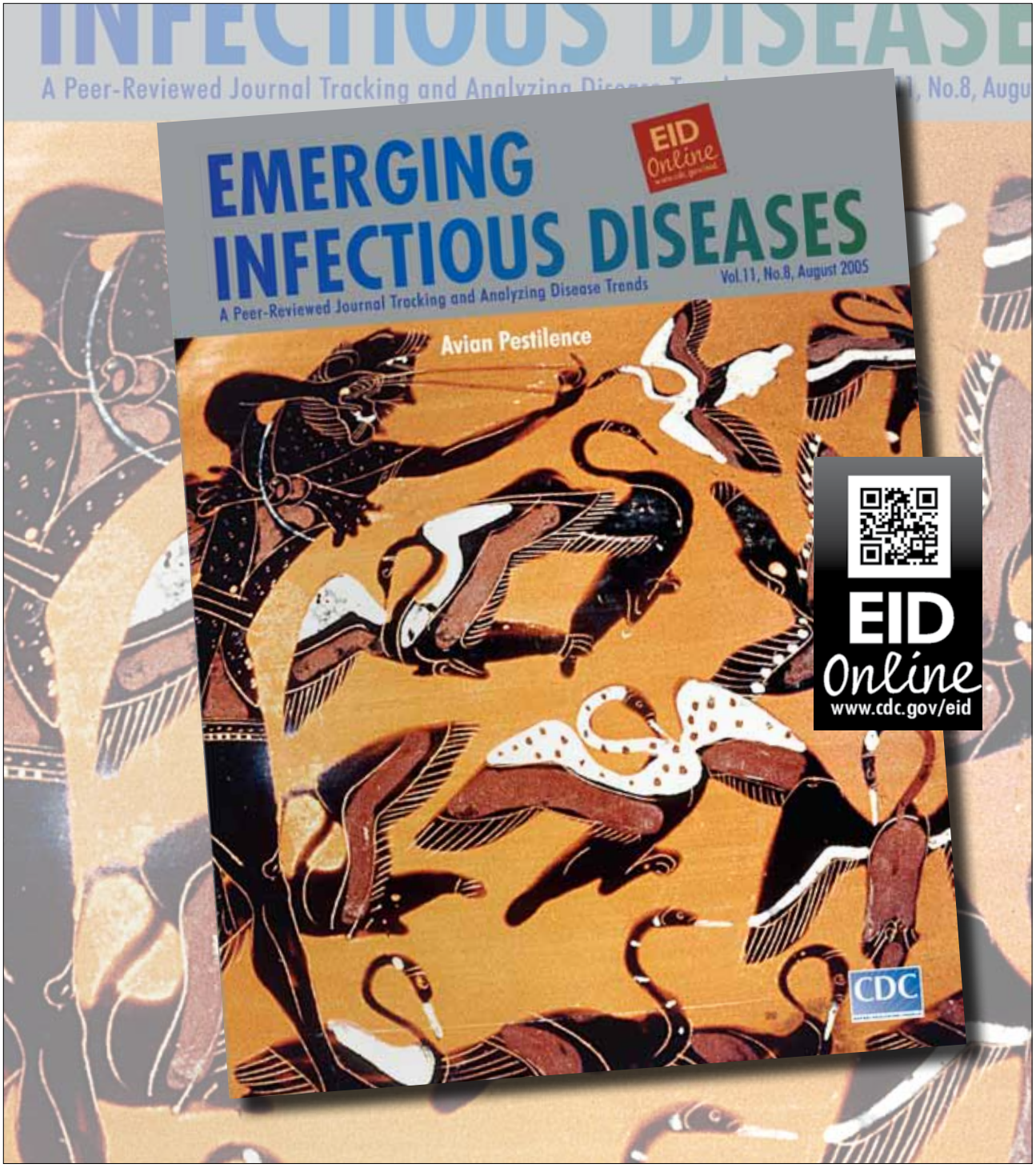

Multidrug-Resistant Atypical Variants of *Shigella flexneri* in China

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We identified 3 atypical *Shigella flexneri* varieties in China, including 92 strains with multidrug resistance, distinct pulse types, and a novel sequence type. Atypical varieties were prevalent mainly in developed regions, and 1 variant has become the dominant *Shigella* spp. serotype in China. Improved surveillance will help guide the prevention and control of shigellosis.

Each year worldwide, ≈1.5 million children <5 years of age die from diarrheal diseases (*1*), which is of particular concern in developing countries. *Shigella* spp. are leading bacterial causes of diarrhea, responsible for ≥80 million cases of bloody diarrhea and 700,000 deaths each year (*2*), and *S. flexneri* serotype 2a has been recognized as the most prevalent serotype in China for many years (*3*). To better understand the etiology of bacterial diarrhea in China and to determine if *S. flexneri* serotype 2a is still the most prevalent serotype in China, we conducted a study during May 2008–December 2010.

The Study

A total of 10,021 fecal samples were obtained from patients with diarrhea or dysentery at hospitals in 8 provinces within the eastern, southern, western, northern, and central regions of China: Liaoning, Shandong, Jiangsu, Guangdong, Gansu, Sichuan, Xinjiang, and Hebei Provinces. Samples were screened for the presence of *Shigella spp*. by using API 20E strips (bioMérieux, Marcy l'Etoile, France). Serotyping was performed by using 1) an antisera kit specific for all type- and group-factor antigens (Denka Seiken, Tokyo, Japan) and 2) a panel of monoclonal antibodies against *S. flexneri* (MASF; Reagensia AB, Stockholm, Sweden). Antimicrobial susceptibility testing was performed by the disk diffusion method as described (*4*). Genetic relationships were estimated by using multilocus sequence typing, as described (*5*), and pulsed-field gel electrophoresis (PFGE) (*6*).

Overall, 1,109 bacterial pathogens were identified. *Shigella* spp. isolates were the most prevalent, representing 273 (24.6%) of the total. Among the *Shigella* strains, 92 atypical strains, with 3 different serologic agglutination profiles, were identified (Table 1); 1 of these strains was initially identified as the previously described serotype 4c (*7*) because it agglutinated with monovalent anti-IV type antisera and monovalent anti-7,8 group antisera. This serotype was also serologically indistinguishable from *S. flexneri* serotype X variant (SFxv), which was recently reported in China (*8*), because it reacted with MASF B–, MASF 7(*8*)–, and MASF IV-1 E1037–specific antibodies (see online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/7/11-1221-Techapp1.pdf). However, this serotype could not produce indole, whereas SFxv could. This atypical serotype was provisionally designated *S. flexneri* X variant (−:7,8, E1037), indole-negative variety. We isolated 73 isolates belonging to this atypical variant, which has become the most frequent serotype in China (Table 1). In 2008, the first 19 *S. flexneri* X variant (−:7,8, E1037) isolates were detected in Beijing; then in 2009, 26 were detected in Beijing, and in 2010, 23 were detected in Beijing, 3 in Jiangsu Province, and 2 in Shandong Province.

The second atypical serotype reacted with monovalent anti-IV type antisera but not the group-specific antisera, and it agglutinated with MASF B–specific and MASF IV-1 E1037–specific antibodies. The serotype was provisionally named *S. flexneri* untypeable variant (−:E1037). Only 2 isolates of this variant were identified in 2010: 1 was recovered from Beijing (9), and the other was recovered from Jiangsu Province.

The third atypical serotype was provisionally designated *S. flexneri* serotype 2 variant (II:3,4,7,8) because it could agglutinate with monovalent anti-II type antisera and monovalent anti-3,4 and anti-7,8 group antisera. It also reacted with type-specific antibody MASF II and group-specific antibodies MASF Y-5 and MASF 7,8 (online Technical Appendix Table 1). A total of, 17 isolates of serotype 2 variant (II:3,4,7,8) have been detected in Jiangsu Province: 10 in 2008, 4 in 2009, and 3 in 2010.

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[1]These authors contributed equally to this article.

Table 1. Distribution of different *Shigella* spp. in China during 2008–2010 and 1991–2000

Serotype	1991–2000* No. (%) isolates	May 2008–December 2010 No. (%) isolates
S. flexneri	8,082 (86.2)	208 (76.2)
1a	272 (2.9)	9 (3.3)
1b	325 (3.5)	29 (10.6)
1c	15 (0.2)	0
2a	6,468 (69.0)	64 (23.4)
2b	152 (1.6)	3 (1.1)
3a	255 (2.7)	0
3b	57 (0.6)	0
3c	9 (0.1)	0
4	230 (2.5)	0
5	30 (0.3)	1 (0.4)
6	73 (0.8)	3 (1.1)
x	70 (0.7)	5 (1.8)
y	126 (1.3)	2 (0.7)
X variant (–:7,8, E1037), indole-negative variety	0	73 (26.7)
S. flexneri serotype 2 variant (II:3,4,7,8)	0	17 (6.2)
S. flexneri untypeable variant (–:E1037)	0	2 (0.7)
S. sonnei	1,022 (10.9)	56 (20.5)
S. dysenteriae	132 (1.4)	6 (2.2)
S. boydii	139 (1.5)	3 (1.1)

*Data are from (*1*).

The atypical serotype strains were all multidrug resistant to ampicillin, ampicillin/sulbactam, chloramphenicol, nalidixic acid, and tetracycline; a total of 91.3% were also resistant to amoxicillin/clavulanic acid, and 84.8% were resistant to trimethoprim/sulfamethoxazole (Table 2; online Technical Appendix Table 2). The atypical strains also showed reduced susceptibility to fluoroquinolones and third-generation cephalosporins. Of the X variant (−:7,8, E1037), indole-negative variety strains, 82.2% were resistant to norfloxacin, 39.7% to ciprofloxacin, 21.9% to levofloxacin, and 13.7% to cefotaxime. Of the serotype 2 variant (II:3,4,7,8) strains, 29.4% were resistant to norfloxacin, 29.4% to ciprofloxacin, 17.6% to levofloxacin, and 17.6% to cefotaxime. The 2 *S. flexneri* untypeable variant (−:E1037) strains were also resistant to norfloxacin and ciprofloxacin. Furthermore, 6 of the X variant (−:7,8, E1037), indole-negative variety strains were also resistant to ciprofloxacin and cefotaxime.

Multilocus sequence typing identified all of the variant strains as a new sequence type (ST), ST100, which differs from ST18 at the lysP locus only (online Technical Appendix Figure). Other serotypes (e.g., 1a, 2a, 2b, and Y) were also identified as ST100. The 73 isolates of X variant (−:7,8, E1037), indole-negative variety were typed into 29 distinct pulse types (PTs) by PFGE analysis, and the 2 *S. flexneri* untypeable variant (−:E1037) isolates belonged to different PTs and had relatively low similarity (online Technical Appendix Figure). The 17 serotype 2 variant (II:3,4,7,8) isolates grouped into 17 PTs, showing high genetic diversity. Several serotype 2 variant (II:3,4,7,8)

Table 2. Antimicrobial drug resistance profiles of variant serotypes of *Shigella flexneri* isolates recovered from patients with diarrhea, China, May 2008–December 2010*

	% Resistant isolates								
	X variant (–:7,8, E1037), indole-negative variety, n = 73			Serotype 2 variant (II:3,4,7,8), n = 17			Untypeable variant (–:E1037), n = 2		
Antimicrobial drug	R	I	S	R	I	S	R	I	S
Ampicillin	100.0	0	0	100.0	0	0	100.0	0	0
Ampicillin/sulbactam	100.0	0	0	100.0	0	0	100.0	0	0
Chloramphenicol	100.0	0	0	100.0	0	0	100.0	0	0
Nalidixic acid	100.0	0	0	100.0	0	0	100.0	0	0
Tetracycline	100.0	0	0	100.0	0	0	100.0	0	0
Amoxicillin/clavulanic acid	91.8	5.5	2.7	88.2	0	11.8	100.0	0	0
Trimethoprim/sulfa	90.4	0	9.6	58.8	0	41.2	100.0	0	0
Norfloxacin	82.2	16.4	1.4	29.4	0	70.6	100.0	0	0
Ciprofloxacin	39.7	49.3	11.0	29.4	0	70.6	100.0	0	0
Levofloxacin	21.9	26.0	52.1	17.6	11.8	70.6	0	50.0	50.0
Cefotaxime	13.7	13.7	72.6	17.6	5.9	76.5	0	0	100.0
Gentamicin	6.8	0	93.2	5.9	0	94.1	0	0	100.0
Ceftazidime	5.5	2.7	91.8	0	0	100.0	0	0	100.0
Imipenem	2.7	0	97.3	0	0	100.0	0	0	100.0

*R, resistant; I, intermediate; S, susceptible.

isolates had greater genetic distance from other serotype 2 isolates. In particular, PT70 had a genetic similarity of <65% with other serotype 2 variant (II:3,4,7,8) isolates.

Conclusions

Three varieties of *S. flexneri* serotypes, including 92 atypical strains, were identified in this study. The X variant (−:7,8, E1037), indole-negative variety displayed a serologic agglutination profile indistinguishable from that for SFxv, but the indole-negative variety had different biochemical reactions, more serious drug resistance, and different ST and PTs. All variant strains were identified as ST100, a new ST containing multiple other serotypes (e.g., 1a, 2a, 2b, and Y). This finding demonstrates that ST100 is the predominant ST circulating among different *S. flexneri* serotypes in China. Moreover, the 3 atypical varieties were also genetically distinct by PFGE analysis and showed a relatively high level of genetic diversity; thus, these *S. flexneri* varieties may have existed for a long time or experienced frequent genetic mutations.

The X variant (−:7,8, E1037), indole-negative variety serotype has supplanted serotype 2a and S. sonnei (3) and represents a new dominant *S. flexneri* serotype in China; serotype 2 variant (II:3,4,7,8) has become the fifth most common serotype of *Shigella* spp. in China. The variant strains were prevalent mainly in Beijing and in Jiangsu and Shandong Provinces; these provinces, located in the eastern and northern regions of China, have more developed economies and larger populations compared with the provinces in western China. Industrialization, trade, frequent movement of population, and environmental change can cause the shifting prevalence of diarrheal pathogens and lead to the dissemination of foodborne diseases worldwide (*10,11*). Therefore, the dynamic change in *S. flexneri*, especially the emergence of new serotypes, may be attributable to the economy and trade development or to human migration.

Of particular concern is that the atypical strains were completely resistant to several antimicrobial drugs used to treat shigellosis in China (*12*), and they showed reduced susceptibility to fluoroquinolones and third-generation cephalosporins. The World Health Organization has recommended ciprofloxacin as a first-line antimicrobial drug for shigellosis treatment (*12*), and third-generation cephalosporins are considered as alternative drug treatments (*13*). However, the *S. flexneri* resistance to ciprofloxacin and cefotaxime that we detected in this study was higher than previously reported in China (*8,14*); this finding raises serious questions regarding the effective treatment of shigellosis in the future.

In conclusion, 3 atypical *S. flexneri* serotypes with extensive multidrug resistance, distinct PTs, and a novel ST were identified in China. Continuous surveillance should be encouraged to determine the changing trends of these variants in geographic, temporal, phenotypic, and genotypic patterns. Such knowledge will improve our understanding of the actual level of disease and provide guidance for the prevention and control of shigellosis.

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MDR TB Transmission, Singapore

To the Editor: Over the past decade, the proportion of pulmonary multidrug-resistant tuberculosis (MDR TB) cases among Singapore-born patients remained low, whereas that among foreign-born patients was 10 times higher (*1*,*2*). Since 2005, Singapore has experienced a sharp increase in the number of MDR TB cases from high-prevalence countries (*3*). We report local transmission of MDR TB in 2011, from a short-stay visitor to 2 Singapore-born persons in a correctional setting.

The index case-patient was a 34-year-old Burmese man (patient A) arrested 10 months after entering Singapore. A screening radiograph taken 2 days after arrest showed a right upper lobe cavitary lesion. The man was referred to the TB Control Unit. He had been coughing for 3 months but had no other concurrent conditions. When the abnormal radiograph results became known, the man was isolated within the prison. Sputum was collected, and first-line anti-TB drugs were administered pending sputum results. The sputum smear had 3+ acid-fast bacilli (AFB); mutations of the *rpoB* and *katG* genes were indicated by testing with GenoType MTDR*plus* (Hain Lifescience, Nehren, Germany). The patient's treatment regimen was modified accordingly; appropriate second-line anti-TB treatment was started 14 days after he entered the institution. *Mycobacterium tuberculosis* complex (MTC) grew from sputum in 9 days; phenotypic drug-susceptibility testing (DST) demonstrated resistance to rifampin, isoniazid, streptomycin, and ethambutol and susceptibility to pyrazinamide, ethionamide, kanamycin, and ofloxacin.

One month after patient A was arrested, a Singapore-born man (patient B) in a public hospital received a diagnosis of HIV infection (67 CD4 cells/mL) and *Pneumocystis jirovecii* pneumonia. He was not an identified contact of patient A, although his job entailed accompanying prisoners from remand centers to justice courts. Antiretroviral treatment (ART) given 1 month after HIV diagnosis resulted in fever 7 days later. A repeat chest radiograph showed increased opacities in the left upper zone. Sputum smear was 4+ for AFB, and MTC with *rpoB* gene mutation was detected (Xpert MTB/RIF; Cepheid, Sunnyvale CA, USA). Second-line anti-TB drugs were administered. MTC was grown in sputum and blood in 14 and 32 days, respectively; phenotypic DST 8 weeks later showed a susceptibility profile that was identical (except for ethambutol susceptibility) to that of patient A.

Patient C was a 43-year-old Singapore-born man arrested 1 month after receiving an HIV diagnosis and beginning ART. He withheld his HIV status from prison authorities. He shared a cell with patient A for ≈48 hours at the remand center before the chest radiograph for patient A was taken. Patient C was released after 2 days and screened as a contact 2 months later; CD4 count was <20 cells/mL despite ART. Physical examination showed peripheral lymphadenopathy. T-SPOT.TB test (Oxford Immunotec Ltd., Abingdon, UK) result was negative, chest radiographs were unremarkable, and 2 sputum smears were negative for AFB (corresponding specimens for TB culture were negative). These findings were communicated to the patient's primary physician. He was hospitalized 3 months later with fever and cough for 5 days but discharged himself, against medical advice, after 2 days. After 11 days, he was readmitted in septic shock to another hospital, at which time his sputum smear was positive for AFB and chest radiograph showed an increased right paratracheal stripe with right lower zone opacities. A bronchoesophageal fistula was also diagnosed, for which he declined intervention. Isoniazid, ethambutol, pyrazinamide, and rifabutin (his second-line ART regimen was incompatible with rifampin) were prescribed, and he discharged himself, against medical advice. After 5 days, he was readmitted with worsening cough; second-line anti-TB medications were instituted when his sputum specimen results were reported 8 weeks later as being phenotypically resistant to rifampin, isoniazid, and streptomycin. DST results for ethambutol were discrepant for isolates cultured from 2 sputum specimens and tested in 2 laboratories.

DNA genotyping by spoligotyping (Ocimum Biosolutions, Hyderabad, India) (*4*) showed that the isolates from all 3 patients belonged to type 467 H3, according to the SITVIT2 database (www.pasteur-guadeloupe.fr:8081/SITVITDemo/). Mycobacterial interspersed repetitive units–variable number tandem repeat genotyping with a 24-loci panel (Genoscreen, Lille, France) (*5*) similarly showed identical profiles (Figure). No other isolates in our database had matching profiles.

The cases reported here echo previous institutional outbreaks of MDR TB in industrialized countries (*6*–*8*). They are a reminder of the potentially devastating consequences when HIV and MDR TB intersect and the need for infection control measures where vulnerable and/or high-risk groups congregate. For patients A and B, rapid genotypic DST expedited the MDR TB diagnosis and institution of appropriate treatment and isolation measures and curtailed further spread. The unmasking immune reconstitution inflammatory syndrome that developed in patient B exemplifies the need for TB screening before starting ART in patients from countries with medium-to-high TB prevalence. For patient C, the several-week delay in instituting second-line TB medications could have been avoided had hospital

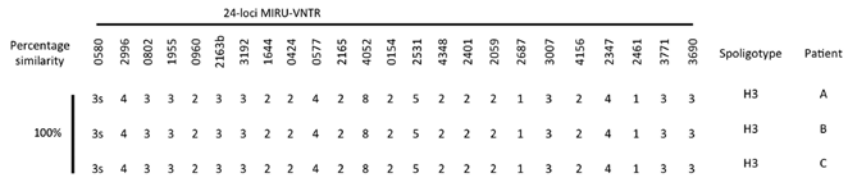

Figure. Spoligotype and 24-loci MIRU-VNTR typing results for *Mycobacterium tuberculosis* complex isolates recovered from 3 patients with multidrug-resistant tuberculosis (TB). Patient A (index case-patient), Burma-born man with TB, incarcerated in Singapore correctional facility; patient B, Singapore-born man with HIV infection and TB, who transported prisoners in Singapore; patient C, Singapore-born man with HIV infection and TB, who shared cell with patient A. MIRU-VNTR, mycobacterial interspersed repetitive units–variable number tandem repeat.

medical teams been aware of his recent MDR TB exposure.

A recent update documented the highest rates of global MDR TB in 2009 and 2010 (*9*). Our experience reported here underscores the need to be constantly mindful of this infectious disease threat in our increasingly borderless world, even in countries where incidence of MDR TB is low.

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Human Infection with Marten Tapeworm

To the Editor: Cysticercosis-like human infections with the tapeworm *Taenia crassiceps*, which infects foxes as terminal hosts, have been reported (*1*,*2*). We report a case of a cysticercosis-like eye infection caused by the tapeworm *T. martis* (marten tapeworm) in a woman.

The patient was a 43-year-old German woman who sought care during July 2010, after 4 days of perceiving flashing lights in her visual field and a paracentral scotoma in her left eye. Visual acuity in both eyes was 20/20. Examination of the left fundus revealed a mobile subretinal tumor at the temporal upper retinal branch vessel with adjacent intraretinal and subhyaloid bleeding (Figure, panels A–C; online Video, wwwnc.cdc.gov/EID/article/19/7/12-1114-F1.htm). The subretinal tumor resembled a cestode larva.

The patient reported no other symptoms at that time. Laboratory evaluation found no eosinophilia or elevation of total IgE. Serologic testing results were negative for antibodies against the following parasites: *Taenia solium*, *Echinococcus multilocularis, E. granulosus, Dirofilaria immitis, Strongyloides* spp., and *Toxocara canis*. Fecal testing results were negative for worm eggs. Images from ultrasonography of the liver and magnetic resonance imaging of the head were unremarkable. The patient's travel history included—in addition to southern European countries—trips to Nepal and Thailand 15 years previously.

At the time of examination, the patient lived in a small village near Freiburg (im Breisgau) in southwestern Germany. She grew vegetables in the family garden, which was next to a forest. Her 3 children and husband did not report any health problems. For the past 6 years, the family had owned

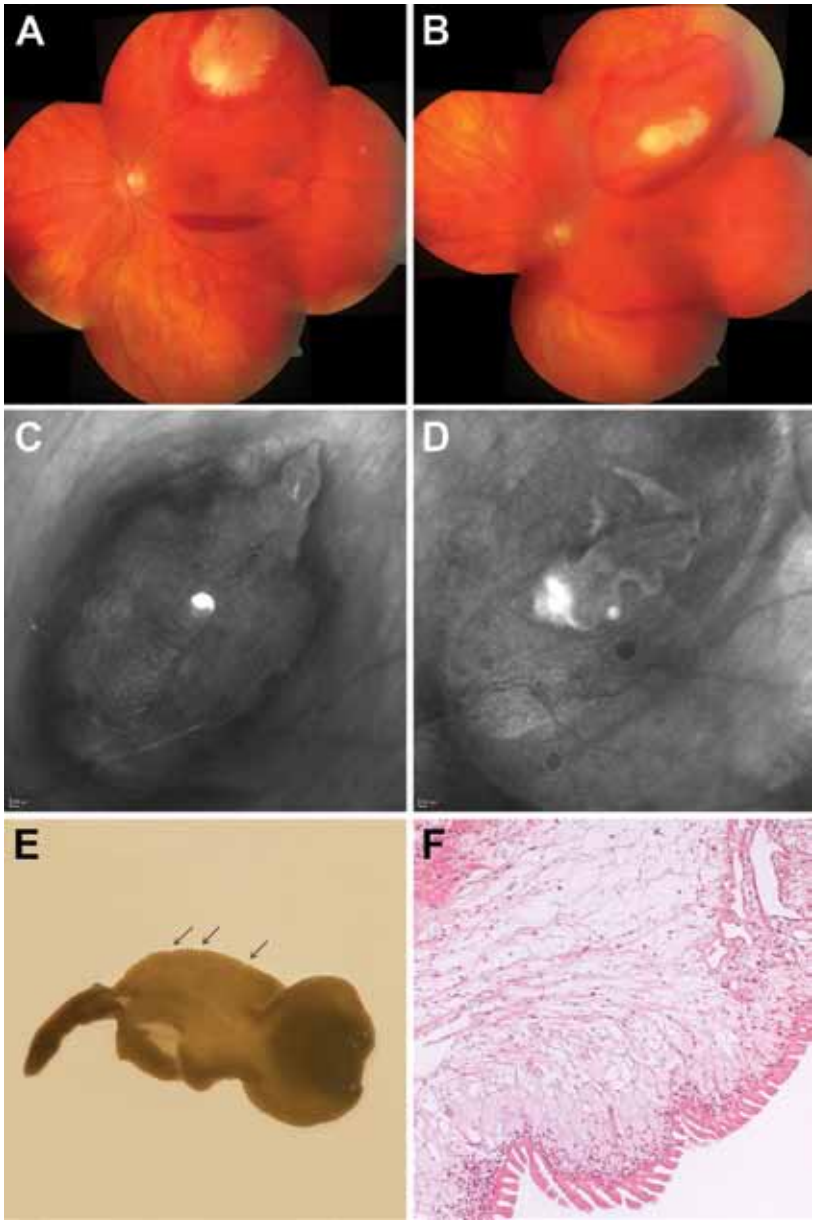

Figure. Cysticercosis-like eye infection caused by the tapeworm *Taenia martis* in a woman. A) Fundus at the patient's initial visit, before medical therapy. The cyst lies subretinally at the temporal upper branch vessels; adjacent intraretinal and subretinal bleeding and central subhyaloid bleeding can be seen. B) After 8 days of medical therapy, the cyst size had decreased markedly. The physis of the larva (A and B) is reminiscent of the armatetrathyridium (or fimbriocercus), a larval form typical for the tapeworm subspecies *T. martis martis.* C) Cyst at patient's initial visit. D) Cyst at the time of surgery. E) Surgically removed monocephalic cysticercus-like larva with inverted parenchymatous portion, withdrawn scolex, and attenuated posterior end. The tegumental surface is transversely striated and exhibits inward folds (arrows). F) Histologic section of the *Taenia martis* tapeworm cyst showing morphologic characteristics also commonly seen in cysticercosis cysts caused by *T. solium* tapeworms. The syncytial bladder wall consists of a rugate external, a nucleated intermediate, and an internal reticular layer with lacunate branches of the excretory duct system. Filamentous extensions of contractile muscles project into the parenchyma, which is interspersed with a few calcareous corpuscles. In addition, the *T. martis* cyst shows a preponderance of uniformly organized, elongate and slender tegumental processes, which are usually not seen in histologic sections of cyst walls caused by *T. solium* tapeworms. Hematoxylin and eosin stain; objective magnification ×10.

a dog, which received antiparasitic medications on a regular basis; recent checks for intestinal parasitic infection found no ova.

The suspected cause of the woman's illness was cysticercosis caused by the larva of *T. solium*; systemic antiparasitic therapy was started (albendazole 400 mg 2×/d, dexamethasone 20 mg/d). The size of the larva diminished (Figure, panel D; Video), but the patient remained symptomatic. Therefore, after 8 days of therapy, the cyst was removed by retinotomy. A few days later, peripheral retinal detachment occurred and was treated by a second vitrectomy and intravitreal gas injection. Because of the repeated gas tamponade, a gas cataract developed, which necessitated cataract surgery. At the end of March 2011, the patient's visual acuity had returned to 20/20 in both eyes.

The removed cyst showed the characteristic macroscopic and histologic features of a cysticercus bladder wall (Figure, panels E, F). To determine the exact species by using molecular methods, we isolated DNA from the cyst, conducted different PCRs selective for mitochondrial genes, determined the corresponding sequences, and used a BLAST search (*3*) to compare these sequences with publically available sequences. Sequences of the following mitochondrial genes were determined by using the given primers and later submitted to GenBank: small ribosomal subunit (primers 12S *Taenia* FF 5′-CACAGTGCCAGCATCYGCGGT-3′ and 12S *Taenia* RR 5′-GAGGGTGACGGGCGGTGTGTAC-3′, PCR product of 426 bp, GenBank accession no. JX415820); NADH dehydrogenase subunit 1 (primers: NAD1-FF 5′-ATTGGKTTATTTCAGAGTTTTTCTGATTTA-3′ and NAD1-RR 5′-CTCMCCATAATCAAATGGACTACG-3′, 394 bp, GenBank accession no. JX415819); and the cytochrome-*c* oxidase subunit 1 (determined by using previously published primers

[*4*,*5*]; 376 bp, GenBank accession no. JX415821). All sequences showed highest identity with *T. martis* (99%–100%) but substantially lower identity with *T. crassiceps* (91%–97%) and *T. solium* (87%–89%) tapeworms.

Thus, molecular methods unequivocally identified the larva as that of a *T. martis* tapeworm. *T. martis* tapeworms (cestodes) live and produce eggs in the intestines of definitive hosts, weasels (family *Mustelidae*), which also includes pine martens, stone martens, polecats, badgers, wolverines, and stoats (*6*). The intermediate hosts are prey animals of the definitive hosts, such as arvicoline (voles and muskrats) or murid rodents. When intermediate hosts ingest eggs, cysticerci develop in the pleural and peritoneal cavities. *T. martis* tapeworms probably occur worldwide wherever suitable definitive and intermediate hosts are present (*6*,*7*). A study in southwest Germany reported that 36% of stone martens were infected with *T. martis* tapeworms (*6*).

Although nearly all patients who had cysticercosis-like infections caused by *T. crassiceps* tapeworms were immunosuppressed (*1*,*2*), we found no signs of immunosuppression in the patient reported here. The only identified risk factor for this patient was consumption of homegrown vegetables, which could have been contaminated by marten feces.

The clinical and histologic appearance of the organism in this patient suggested cysticercosis caused by a *T. solium* tapeworm. However, the specific diagnosis of *T. martis* tapeworm infection was possible only by use of molecular methods. Thus, human infections with *T. martis* and other animal tapeworms might occur at times but might be misdiagnosed as *T. solium* cysticercosis. For therapy, the rules and considerations are probably the same as those for *T. solium* cysticercosis, as described (*8*,*9*). Concerning antiparasitic therapy, one must be aware of possible complications caused by intraocular immunologic reactions. As demonstrated by the case reported here, surgical removal of a subretinal larva is connected with the risk for retinal detachment and cataract formation. The identification of the responsible tapeworm is useful for epidemiologic reasons, for determining the source of infection. We therefore suggest using molecular methods to determine the exact species of parasites removed from human tissue.

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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.

Campylobacter jejuni in Hospitalized Patients with Diarrhea, Kolkata, India

To the Editor: *Campylobacter* spp. infection is the leading cause of bacterial enteritis worldwide. The epidemiology of *Campylobacter* infection in developing countries differs substantially from that in industrialized countries. In many studies from the United States and other industrialized countries, *Campylobacter* spp. are among the most common bacterial causes of diarrhea (*1*), with an incidence of ≈10% in persons with diarrhea. Reports from developing countries also suggest that *C. jejuni* and *C. coli* have been isolated mostly from patients with diarrheal illness (*2*). We investigated for the prevalence of *Campylobacter* infection in patients hospitalized with diarrhea at the Infectious Disease Hospital in Kolkata, India, and their resistance patterns to different antimicrobial drugs.

During January 2008–December 2010, we screened 3,186 fecal samples on brain–heart infusion agar with 5% defibrinated sheep blood and antimicrobial drugs (bacitracin, cycloheximide, colistin sulfate, cephazoline sodium, novobiocin) and incubated under microaerophillic environment (5% O_2, 10% CO_2, and 85% N_2) at 37°C for 48 h. Each isolate was tested by Gram staining, cytochrome oxidase, and hippurate hydrolysis for presumptive identification and species-specific PCR (*3*) to identify 5 species from *Campylobacter* genus. The overall isolation rate of *Campylobacter* spp. was ≈7% (222/3,186). Sole infection with *Campylobacter* spp. accounted for only 40% of cases; others were mixed infection. *C. jejuni* was the predominant species (78%), with *C. coli*, *C. fetus*, *C. lari*, and *C. upsaliensis* isolated less frequently. *Campylobacter* infection prevailed throughout the year, with no seasonality. The *C. jejuni* isolation rate was significantly higher (10.0%; $p<0.001$) for children <5 years of age who had diarrhea than for persons in other age groups (3.7%). Although we used the culture method, which is the standard for screening fecal samples, some molecular methods, such as PCR and real-time PCR, are now used for screening *Campylobacter* spp. from fecal samples on the Indian subcontinent (*4*,*5*). The results from molecular methods are showing more infection with *Campylobacter* spp. and high mixed infection cases and suggest the usefulness of molecular methods in combination with cultures.

Macrolides and fluoroquinolones generally are the first- and second-line choices, respectively, of antimicrobial drugs for treating *Campylobacter* enteritis. Since the late 1980s, resistance to these drugs has complicated treatment. In India, resistance of *Campylobacter* spp. to several antimicrobial drugs has been reported since the early 1990s (*6*). Fluoroquinolone resistance was not reported in 1994 but reached 79% during 2001–2006 (*2*). Likewise, ciprofloxacin resistance in *Campylobacter* spp. increased markedly in Dhaka, Bangladesh, during 2005–2008 (*7*) and in Karachi, Pakistan, during 1992–2002 (*8*). We tested 142 *C. jejuni* isolates for antimicrobial susceptibility by disk diffusion method on Muller-Hinton agar with 5% sheep blood and incubated them at 37°C in microaerophillic environment for 48 h before obtaining results. All tested strains were resistant to trimethoprim–sulfamethoxazole, and most (97%) were resistant to quinolone (nalidixic acid) and fluoroquinolones (norfloxacin, ciprofloxacin, and ofloxacin) (Figure). Approximately 26.1% and 17.6% of the isolates were resistant to ampicillin and tetracycline, respectively. Susceptibility to erythromycin, azithromycin, gentamicin, furazolidone, and chloramphenicol was very high (>97%) in most isolates. Multidrug resistance was frequent among many of the isolates: ampicillin, nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, and trimethoprim–sulfamethoxazole (19%); tetracycline, nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, and trimethoprim–sulfamethoxazole (10.2%); and tetracycline, ampicillin, nalidixic acid, norfloxacin, ciprofloxacin, ofloxacin, and trimethoprim–sulfamethoxazole (6.8%). These results indicate that macrolides may be useful for treating campylobacteriosis in this region.

The resistance patterns are influenced by various factors, possibly including pressure exerted by use of antimicrobial drugs. Various reports

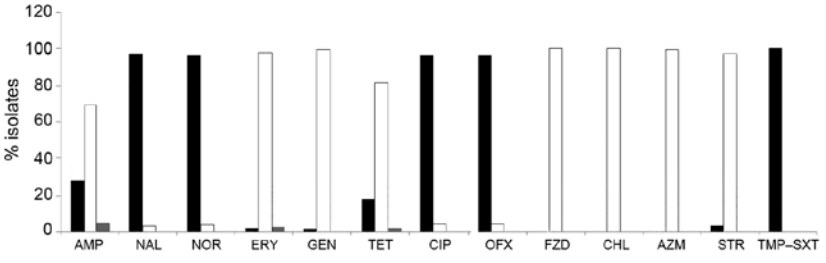

Figure. Antimicrobial drug susceptibility profile of 142 *Campylobacter jejuni* isolates, Kolkata, India, 2008–2010. Black bars, resistant; gray bars, intermediate resistance; white bars, susceptible. AMP, ampicillin; NAL, nalidixic acid; NOR, norfloxacin; ERY, erythromycin; GEN, gentamicin; TET, tetracycline; CIP, ciprofloxacin; OFX, ofloxacin; FZD, furazolidone; CHL, chloramphenicol; AZM, azithromycin; STR, streptomycin; TMP–SXT, trimethoprim–sulfamethoxazole.

have stated that introduction of fluoroquinolones for use in veterinary practice has been associated with a dramatic rise in *Campylobacter* strains showing resistance to these drugs (*9*). Increasing antimicrobial drug resistance limits the number of therapeutic options, which makes empirical treatment more difficult. Therefore, constant monitoring of *Campylobacter* susceptibility to antimicrobial agents is essential. We could not detect any allele of plasmid-mediated quinolone resistance genes (*qnr*) among *C. jejuni* isolates and the different class of mobile genetic elements that generally carry the antimicrobial resistance gene cassettes. However, we found that most of the *C. jejuni* isolates had a mutation in the quinolone-resistance determining region of *gyrA* (Thr-86 to Ile), which led the isolates to become resistant for quinolone and fluoroquinolones.

Recent microbiome analysis of the gut of a malnourished child residing in an urban slum in Kolkata showed 35 times more *Campylobacter* bacteria than in healthy child in the same setting (*10*). This finding suggests that intestinal inflammation may directly influence malabsorption of nutrients. Hence, it is essential to examine the effect of *Campylobacter* infection in the developing world in the context of many recent developments in the human gut microbiome.

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Verona Integron–encoded Metallo-β-Lactamase 1 in Enterobacteria, Ontario, Canada

To the Editor: Among *Enterobacteriaceae*, Verona integron–encoded metallo-β-lactamase 1 (VIM-1) has been found only in *Klebsiella pneumoniae* in North America (*1*). We report 4 VIM-1–producing *Enterobacteriaceae* isolated from 4 patients at 3 hospitals in Ontario, Canada.

Patient 1, a 61-year-old man, was initially hospitalized in Italy for presumed pneumonia and was treated with levofloxacin during his 6-month stay in Italy. Upon returning to Ontario, Canada, he was admitted to hospital 1 in August 2010 because of diabetic ketoacidosis and began empiric treatment with metronidazole and gentamicin. Urine cultures were positive for a carbapenem-resistant *Escherichia coli* (strain GN531). Two days later, the patient had a fever and a blood culture was positive for *E. coli* (strain GN532), which was also resistant to carbapenems. During his hospitalization, the patient was isolated and received droplet precaution because of his travel history until he was discharged home.

Patient 2, a 76-year-old man, was admitted to hospital 2 in May 2011 because of a recurrent urinary tract

infection (urine was positive for *E. coli*). The patient was given ciprofloxacin. On day 49, a carbapenem-sensitive *Enterobacter cloacae* was isolated from urine. On day 61, a carbapenem-resistant *E. cloacae* was isolated from urine culture (strain GN719). Contact precautions were used until the patient was discharged to a long-term care facility on day 80.

Patient 3, an 81-year-old man, was admitted to hospital 2 (November 2011) 2 months after patient 2 was discharged. Urine culture at admission was positive for a carbapenem-resistant *E. cloacae* (strain GN825). The patient was given ceftriaxone and metronidazole and then given ertapenem. The patient died on day 110. Patients 2 and 3 had no hospital room in common during their admissions and both received contact precautions for methicillin-resistant *Staphylococcus aureus* before isolation of the carbapenem-resistant isolates.

Patient 4, a 90-year-old woman, was admitted to hospital 3 in November 2011 because of nausea, vomiting, and diarrhea. In the preceding 6-month period, she had recurrent *Clostridium difficile*–associated diarrhea and a urinary tract infection. At admission, a carbapenem-susceptible *Proteus* spp. was isolated from a urine culture. The patient was given a 3-day course of ciprofloxacin and vancomycin. On day 17, a carbapenem-resistant *E. cloacae* was isolated from urine (strain GN738). Because this organism was also isolated from a rectal swab specimen, it was assumed that the urine sample might be contaminated by her feces. Therefore, the patient did not receive additional treatment other than that for recurrent *C. difficile*–associated diarrhea.

Patients 2, 3, and 4 had no history of travel outside Canada. All 5

Table. VIM-1–producing *Escherichia coli* and *Enterobacter cloacae* clinical isolates, derivative transconjugants, and transformants, Ontario, Canada*

Characteristic	*E. coli* GN531	*E. cloacae* GN719	*E. cloacae* GN738	*E. cloacae* GN825	*E. coli* J-531	*E. coli* T-719	*E. coli* T-825	*E. coli* Top10	*E. coli* J53
Drug, MIC (mg/L)†									
Ampicillin	≥256	≥256	≥256	≥256	≥256	≥256	≥256	3	6
Cefoxitin	≥256	≥256	≥256	≥256	64	≥256	≥256	6	8
Ceftazidime	≥256	≥256	≥256	≥256	≥256	≥256	≥256	0.19	0.19
Cefotaxime	≥256	≥256	≥256	≥256	96	128	≥256	0.094	0.094
Cefepime	256	32	192	256	12	12	24	<0.016	0.064
Ertapenem	2	2	8	24	0.125	0.25	0.25	0.004	0.008
Meropenem	1.5	6	6	16	0.5	0.5	0.5	0.023	0.023
Imipenem	4	6	6	8	2	2	1.5	0.19	0.38
Aztreonam	≥256	0.19	4	1.5	192	0.125	0.125	0.125	0.125
Amikacin	8	2	3	2	3	1.5	1.5	2	1.5
Gentamicin	96	12	2	96	4	0.75	2	0.064	1.5
Tobramycin	32	48	6	32	12	4	4	0.25	1
Nalidixic acid	≥256	≥256	≥256	≥256	32	2	1	1	3
Ciprofloxacin	≥32	≥32	≥32	≥32	0.5	0.125	<0.002	<0.002	0.012
Levofloxacin	≥8	≥32	≥32	8	0.5	0.094	0.002	0.003	0.016
Tetracycline	≤4	192	2	256	0.5	32	32	0.75	1
Tigecycline	0.094	1	0.5	1	0.047	0.064	0.094	0.032	0.047
Colistin	0.064	0.094	0.094	0.125	0.047	0.023	0.016	0.016	0.047
Co-trimoxazole	≥32	≥32	≥32	≥32	≥32	≥32	0.047	0.023	0.064
Drug resistance gene‡									
bla_{VIM-1}	+	+	+	+	+	+	+	NA	NA
$bla_{CTX-M-15}$	+	–	–	–	–	–	–	NA	NA
bla_{TEM-1}	–	+	–	+	–	–	–	NA	NA
bla_{ACC-1}	–	–	+	+	–	–	–	NA	NA
$bla_{OXA-1\ like}$	+	–	–	–	–	–	–	NA	NA
bla_{SHV-12}	+	–	–	–	+	–	–	NA	NA
qnrS1	+	+	+	–	+	+	–	NA	NA
Replicon type§									
IncN	+	+	+	+	+	+	–	NA	NA
IncFrep	+	+	–	+	–	–	–	NA	NA
IncFIB	+	–	–	+	–	–	–	NA	NA
IncFIA	+	–	–	–	–	–	–	NA	NA

*VIM-1, Verona integron–encoded metallo-β-lactamase 1; *E. coli* J-531, *E. coli* transconjugant derived from GN531; *E. coli* T-719 and T-825, *E. coli* transformants derived from GN719 and GN825, respectively; *E. coli* J53 and TOP10, recipient *E. coli* J53 and TOP10, respectively; bla, β-lactamase; +, positive; NA, not applicable (only genes and replicons detected by molecular screening are included); –, negative; qnr, quinolone resistance; Inc, incompatibility.

†Drug susceptibility results were determined by using Etest (bioMérieux, Marcy l'Etoile, France) and the agar dilution method and interpreted by using Clinical and Laboratory Standards Institute guidelines (*3*).

‡Sequencing of whole genes was performed in samples positive by PCR. PCR included screening for bla_{TEM}; bla_{SHV}; $bla_{OXA-1-like}$; bla_{CTX-M} groups 1, 2, and 9; bla_{VEB}; bla_{PER}; bla_{GES}; $bla_{OXA-48-like}$; bla_{IMP}; bla_{KPC}; bla_{NDM-1}; and 6 groups of bla_{AmpC} genes (*4*).

§Obtained by using the replicon typing approach of Carattoli et al. (*5*).

isolates were submitted for reference purposes to the Public Health Ontario Laboratories. Pulsed-field gel electrophoresis showed that *E. coli* GN531 and GN532 were indistinguishable (GN531 was selected for further studies), and the 3 *E. cloacae* isolates had similar fingerprint patterns. All strains displayed synergy in presence of meropenem disks plus dipicolinic acid, which is indicative of metallo-β-lactamase inhibition (*2*). The 4 clinical strains displayed a multidrug resistance phenotype, and were susceptible only to tigecycline and colistin (Table).

PCR and sequencing identified bla_{VIM-1} in all isolates (Table). Multilocus sequence typing classified *E. coli* GN531 as sequence type (ST) 131 (*6*), the epidemic strain that spreads $bla_{CTX-M-15}$ worldwide (*7*). *E. coli* ST131 with similar phenotypic and genetic features was described in Florence, Italy, in 2009 (*8*). Because *E. coli* GN531 was isolated from patient 1, who had received heath care in Italy before being hospitalized in Ontario, this patient might have been exposed to this strain in Italy. A similar scenario was reported in the first case of VIM-1–producing *K. pneumoniae* in the United States, which was isolated from a patient who received health care in Greece (*1*). The presence of a metallo-β-lactamase in *E. coli* ST131 is of great concern because it increases the potential for dissemination of drug-resistance genes.

An IncN plasmid (*5*) harboring bla_{VIM-1} was transferred from GN531 to *E. coli* by conjugation (Table). The $bla_{CTX-M-15}$ gene was not co-transferred, which indicated that it was located on another plasmid or the chromosome of the clinical isolate. After several attempts, no transconjugants derived from *E. cloacae* were obtained. *E. coli* TOP10 (Life Technologies, Carlsbad, CA, USA) was transformed with VIM-1 plasmids obtained from *E. cloacae* GN719 and GN825 (T-719 and T-825, respectively). *E. coli* transformation with plasmid extracts from *E. cloacae* GN738 was unsuccessful. Pulsed-field gel electrophoresis with S1 nuclease (*9*) and Southern blot analysis identified VIM-1-containing plasmids; estimated sizes were 65 kb (*E. coli* GN531), 50 kb (*E. cloacae* GN738), and 30 kb (*E. cloacae* GN719 and GN825).

In conclusion, VIM-1 was found among *Enterobacteriaceae* from 3 geographically distant nosocomial units in Ontario, Canada. Although *E. cloacae* strains were clonally related, there were no clear epidemiologic links between these patients, suggesting that the clone or resistance gene maybe circulating in the province on a greater scale than believed. Emergence of *E. coli* ST131, a pandemic multidrug-resistant clone that causes predominantly community-onset infections (*7*), and produces simultaneously CTX-M-15 and VIM-1, could be a serious threat for the dissemination of these drug-resistance elements.

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Novel Bat-borne Hantavirus, Vietnam

To the Editor: Compelling evidence of genetically distinct hantaviruses (family *Bunyaviridae*) in multiple species of shrews and moles (order Soricomorpha, families Soricidae and Talpidae) across 4 continents (*1*–*7*) suggests that soricomorphs, rather than rodents (order Rodentia, families *Muridae* and *Cricetidae*), might be the primordial hosts (*6*,*7*). Recently, the host range of hantaviruses has been further expanded by the discovery that insectivorous bats (order Chiroptera) also serve as reservoirs (*8*,*9*). Conjecturing that Mouyassué virus in the banana pipistrelle (*Neoromicia nanus*) in Côte d'Ivoire (*8*) and Magboi virus (MGBV) in the hairy split-faced bat (*Nycteris hispida*) in Sierra Leone (*9*) represent a much broader geographic distribution of bat-borne hantaviruses, we analyzed tissues from bats captured in Mongolia and Vietnam.

Total RNA was extracted from 51 lung tissues, collected in RNAlater Stabilization Reagent (QIAGEN, Valencia, CA, USA), from insectivorous bats, representing 7 genera and 12 species, captured in Mongolia and Vietnam. cDNA was then prepared by using PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Otsu, Shiga, Japan) for reverse transcription PCR (RT-PCR), and using oligonucleotide primers previously designed for amplification of soricid- and talpid-borne hantaviruses (*1*–*7*).

A novel hantavirus, designated Xuan Son virus (XSV), was detected in 1 of 5 Pomona roundleaf bats (*Hipposideros pomona*) by using a heminested large (L)–segment primer set (outer: HNL-2111F, 5′-CARTCWACWGTIGGIGCIAGTGG-3′, and HAN-L-R1, 5′-AACCADTCWGTYCCRTCATC-3′; inner: HNL-2111F and HAN-L-R2, 5′-GCRTCRTCWGARTGRTGDGCAA-3′) and a nested small (S)–segment primer set (outer: OS-M55F, 5′-TAGTAGTAGACTCC-3′, and XSV-S6R, 5′-AGITCIGGRTCCATRTCRTCICC-3′; inner: Cro-2F, 5′-AGYCCIGTIATGRGWGTIRTYGG-3′, and JJUVS-1233R, 5′-TCACCMAGRTGRAAGTGRTCIAC-3. The bat was captured during July 2012 in Xuan Son National Park, a nature reserve in Thanh Sơn District, Phu Tho Province, ≈100 km west of Hanoi (21°07′26.75′N, 104°57′29.98′′E).

For confirmation, RNA extraction and RT-PCR were performed independently in a laboratory in which hantaviruses had never been handled. After initial detection, the L-segment sequence was extended by using another primer set (PHL-173F: 5′-GATWAAGCATGAYTGGTCTGA-3′; and TNL-5084R: 5′-GATCCTGAARTACAATGTGCTGG-3′). To calculate the number of virus copies in tissues by real-time RT-PCR, we used a virus-specific primer set (XSV-F: 5′-GTTGCACAGCTTGGTATTGG-3′; and XSV-R: 5′-TTAGCACCCAAACCTCCAAG-3′) and probe (XSV-Probe: 5′-ACAGCTCCTGGCATGGTAAATTCTCC-3′).

Pairwise alignment and comparison (with ClustalW, www.clustal.org) of a 4,582-nt (1,527 aa) region of the RNA-dependent RNA polymerase–encoding L segment indicated sequence similarities of 71.4%–71.5% and 75.9%–78.7% at the nucleotide and amino acid levels, respectively, between XSV and Mouyassué virus and MGBV. Sequence analysis of a 499-nt (166 aa) region of the nucleocapsid-encoding S segment showed that XSV differed by 42.8%–58.3% from representative hantaviruses harbored by rodents and most soricomorphs. XSV sequences were identical in lung, liver, kidney, and spleen; and the highest number of virus copies (7.6×10^1) was in lung tissue, determined by real-time RT-PCR. No additional hantavirus-infected Pomona roundleaf bats were found by RT-PCR that used XSV-specific primers.

Phylogenetic analyses was performed with maximum-likelihood and Bayesian methods, and we used the GTR+I+Γ model of evolution, as selected by the hierarchical likelihood-ratio test in MrModel-test version 2.3 and jModelTest version 0.1 (*10*), partitioned by codon position. Results indicated 4 distinct phylogroups, with XSV sharing a common ancestry with MGBV (Figure). Similar topologies, supported by high bootstrap (>70%) and posterior node (>0.70) probabilities, were consistently derived when various algorithms and different taxa and combinations of taxa were used. Moreover, as we reported previously, the incongruence between some hantaviruses and their reservoir hosts might be indicative of host-switching events (*5*–*7*).

The striking sequence divergence of XSV presented considerable challenges for designing suitable primers for RT-PCR and sequencing. Also, sequencing efforts were constrained by the limited availability of tissues and concurrent virus isolation attempts. Consequently, we were unable to obtain the full-length sequence of XSV. Similarly, the inability to detect hantavirus RNA in tissues from other species of bats in this study might be attributed to several factors, including the highly focal nature of hantavirus infection, small sample sizes of bats of any given species, primer mismatches, and suboptimal cycling conditions.

Bats of the genus *Hipposideros,* family Hipposideridae, are among the most speciose insectivorous bats; ≈70 species are distributed across Africa, Europe, Asia, and Australia. Pomona roundleaf bats are frequently found in or near limestone or sandstone caves. Their colony sizes vary from few to many hundreds of individuals. The vast geographic distribution of the Pomona roundleaf bat throughout

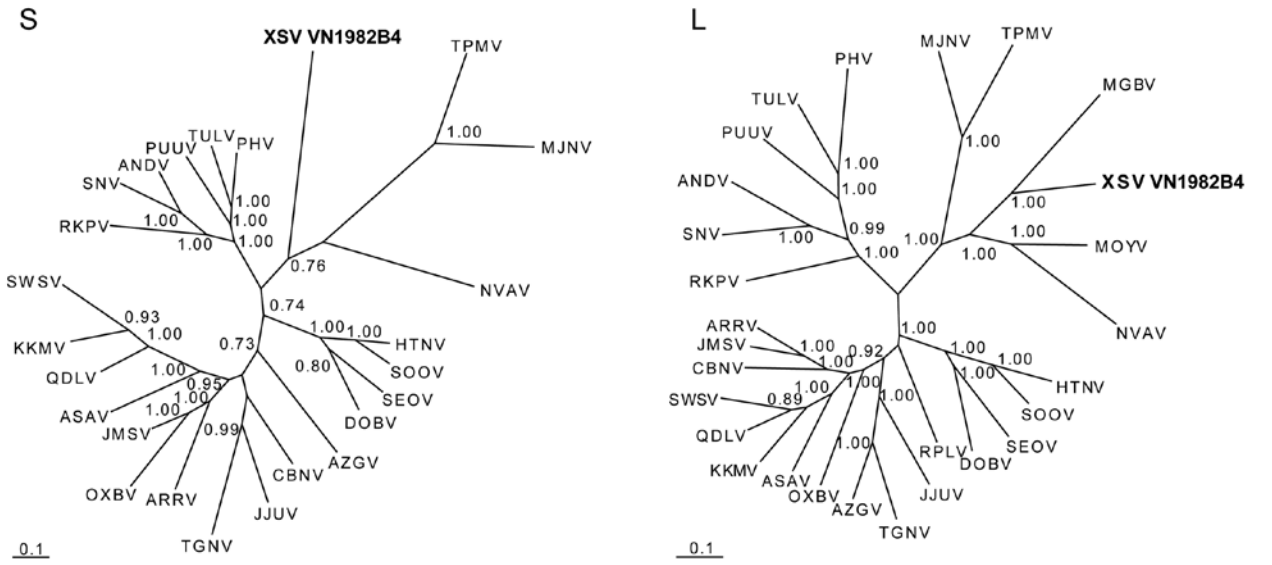

Figure. Phylogenetic trees, based on 499-nt and 4,582-nt regions of the small (S) and large (L) genomic segments, respectively, of Xuan Son virus (XSV VN1982B4) (GenBank accession nos. S: KC688335, L: JX912953), generated by the maximum-likelihood and Bayesian Markov chain Monte Carlo estimation methods, under the GTR+I+Γ model of evolution. Because tree topologies were similar when RAxML and MrBayes were used, the tree generated by MrBayes was displayed. The phylogenetic position of XSV is shown in relation to chiropteran-borne hantaviruses, Mouyassué virus ([MOYV] JQ287716) from the banana pipistrelle and Magboi virus ([MGBV] JN037851) from the hairy slit-faced bat. The taxonomic identity of the XSV-infected Pomona roundleaf bat was confirmed by mitochondrial DNA analysis (GenBank accession no. JX912954). The numbers at each node are Bayesian posterior probabilities (>0.7), and the scale bars indicate nucleotide substitutions per site. **Boldface** indicates the Xuan Son virus detected in Pomona roundleaf bat, Vietnam. Representative soricomorph-borne hantaviruses include Thottapalayam virus ([TPMV] AY526097, EU001330) from the Asian house shrew; Imjin virus ([MJNV] EF641804, EF641806) from the Ussuri white-toothed shrew; Jeju virus ([JJUV] HQ663933, HQ663935) from the Asian lesser white-toothed shrew; Tanganya virus ([TGNV] EF050455, EF050454) from the Therese's shrew; Azagny virus ([AZGV] JF276226, JF276228) from the West African pygmy shrew; Cao Bang virus ([CBNV] EF543524, EF543525) from the Chinese mole shrew; Ash River virus ([ARRV] EF650086, EF619961) from the masked shrew; Jemez Springs virus ([JMSV] FJ593499, FJ593501) from the dusky shrew; Seewis virus ([SWSV] EF636024, EF636026) from the Eurasian common shrew; Kenkeme virus ([KKMV] GQ306148, GQ306150) from the flat-skulled shrew; Qiandao Lake virus ([QDLV] GU566023, GU566021) from the stripe-backed shrew; Camp Ripley virus ([RPLV] EF540771) from the northern short-tailed shrew; Asama virus ([ASAV] EU929072, EU929078) from the Japanese shrew mole; Oxbow virus ([OXBV] FJ539166, FJ593497) from the American shrew mole; Rockport virus ([RKPV] HM015223, HM015221) from the eastern mole; and Nova virus ([NVAV] FJ539168, FJ593498) from the European common mole. Also shown are representative rodent-borne hantaviruses, including Hantaan virus ([HTNV] NC_005218, NC_005222), Soochong virus ([SOOV] AY675349, DQ562292), Dobrava-Belgrade virus ([DOBV] NC_005233, NC_005235), Seoul virus ([SEOV] NC_005236, NC_005238), Tula virus ([TULV] NC_005227, NC_005226), Puumala virus ([PUUV] NC_005224, NC_005225), Prospect Hill virus ([PHV] Z49098, EF646763), Andes virus ([ANDV] NC_003466, NC_003468), and Sin Nombre virus ([SNV] NC_005216, NC_005217).

Vietnam and in Bangladesh, Cambodia, China, India, Laos, Malaysia, Myanmar, Nepal, and Thailand, provides opportunities to ascertain the genetic diversity and phylogeography of XSV and XSV-related hantaviruses. In this regard, although hantavirus RNA was not detected in archival tissues from bats of ≈20 genera, including several other *Hipposideros* species (*8*,*9*), many more genetically divergent hantavirus species are probably harbored by insectivorous bats. Not all orphan viruses warrant intensive study at the time of their discovery. However, insights into the ecology and transmission dynamics of newfound bat-borne hantaviruses might prepare us to more rapidly diagnose future outbreaks caused by emerging hantaviruses.

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Possible Cause of Liver Failure in Patient with Dengue Shock Syndrome

To the Editor: We report a rare hepatic ultrasonograph finding for a patient with liver failure associated with dengue virus (DENV) infection. This finding might shed light on the pathogenesis of liver involvement in this disease.

In March 2006, a 10-year-old previously healthy boy was hospitalized for a 3-day history of fever, headache, and nausea/vomiting. Fever subsided on the day of admission, but the patient was in shock (blood pressure 80/40 mm Hg) and had gastrointestinal bleeding and hematuria. Physical examination showed an obese, confused patient with generalized petechiae and hepatomegaly. The initial diagnosis was dengue shock syndrome (DSS). The patient was intubated and received intravenous fluid infusion, packed red blood cells, ceftriaxone, sodium bicarbonate, and ranitidine before being transferred to King Chulalongkorn Memorial Hospital in Bangkok. The patient's blood pressure increased to 130/90 mm Hg after the initial fluid resuscitation (28 mL/kg free flow), and systolic pressure remained at ≈130 mm Hg until transfer.

Laboratory examinations found 14,930 leukocytes/mm^3, hemoglobin 16.4 g/dL, hematocrit 48.2%, platelet 18,000/mm^3, blood urea nitrogen 33 mg/dL, creatinine 1 mg/dL, sodium 128 mEq/L, potassium 6.2 mEq/L, chloride 91 mEq/L, total CO_2 5 mEq/L, total bilirubin 6.9 mg/dL, direct bilirubin 3.9 mg/dL, aspartate transaminase 3,507 IU/L, alanine transaminase 2,775 IU/L, prothrombin time 43 seconds (international normalized ratio 3.4), and partial thromboplastin time 93.5 s (control 28.7 s). Blood and urine cultures showed negative results. Serum was positive for IgM against DENV. Unfortunately, we did not investigate other viral causes of liver failure.

DSS with liver failure was diagnosed and treated with intravenous fluid, sodium bicarbonate, omeprazole, fresh frozen plasma, platelet transfusion, vitamin K, and recombinant factor VIIa concentrate (NovoSeven; Novo Nordisk, Bagsvaerd, Denmark). Despite stable blood pressure over the next 6 days, liver enzymes continued to rise with progressive jaundice (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/12-1820-Techapp1.pdf). Hepatic ultrasonograph on the second

day after admission showed totally reversed direction of portal venous blood flow away from the liver (Figure, panel A), becoming bidirectional on the following day and, finally, reverting to normal direction (although with low velocity) 3 days later (Figure, panel B). Despite improved hemodynamic status, progressive encephalopathy and gastrointestinal bleeding developed and were unresponsive to treatment. Six days later, the patient died of pulmonary hemorrhage and progressive respiratory failure.

DENV infection is one of the most prevalent emerging infectious diseases affecting children and one of the leading causes of liver failure in tropical countries (*1*,*2*). Although liver involvement in patients with dengue hemorrhagic fever is well known, the mechanism for DENV-induced liver injury is still a mystery. Liver autopsy specimens of terminal DSS patients generally showed massive or focal necrosis with little or no recruitment of polymorphonuclear cells or lymphocytes (*3*,*4*). Ultrasonograph images from patients with liver failure caused by acetaminophen poisoning or hepatitis B indicate increased portal vein flow and normal flow velocity to the damaged liver (*5*). Decreased portal vein flow velocity and reversal of the flow direction is seen in the terminal stage of hepatic cirrhosis and a few other conditions such as hepatic sinusoidal obstruction (hepatic veno-occlusive disease), arterioportal fistula, extrahepatic portal vein thrombosis, and hepatic venous outflow obstruction (*6*). This finding is unusual in other instances of toxin- or virus-induced liver failure and might contribute to the understanding of the mechanism of liver involvement in patients with DENV infection.

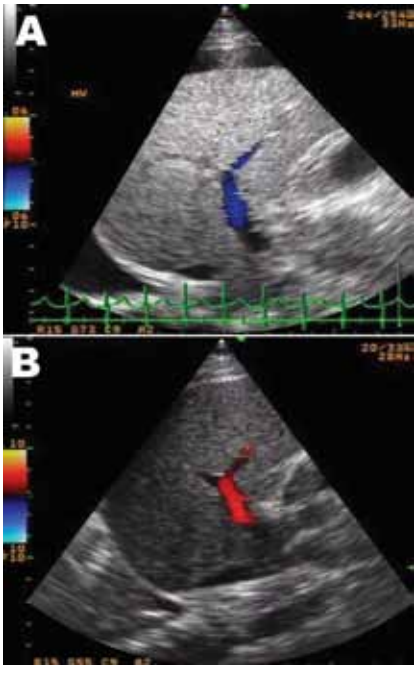

Figure. Ultrasonograph with Doppler image of the liver of a 10-year-old boy with liver failure associated with dengue virus infection. A) Day 2 of hospitalization, showing reversed direction of blood flow in the right branch of the portal vein (hepatofugal flow). There was diffuse increased liver parenchymal echo, swelling of the gallbladder wall, and right pleural effusion. B) Day 5 of hospitalization, showing returning normal direction of portal venous flow (hepatopetal flow). Liver parenchymal echo changed to normal. Pleural fluid and swelling of the gallbladder wall also disappeared.

We previously reported increased portal vein congestion during the toxic stage of DENV infection (*7*). At defervescence, the portal vein was dilated and blood flow velocity was decreased. This finding is usually observed for patients with high resistance in the hepatic sinusoidal capillary network, such as those with liver cirrhosis, and is correlated with the degree of portal venous hypertension (*8*). We postulate that DENV infection of the liver might affect the sinusoidal endothelial or Kupffer cells in a way that causes obstruction to the hepatic sinusoidal capillary lumen resulting in decreased portal venous blood velocity and flow to the liver and, when severe, shunting of portal blood away from the liver (hepatofugal flow). Because portal venous blood comprises 75% of total hepatic blood (*6*), this condition coupled with decreased hepatic arterial blood flow as a consequence of shock might have led to severe and irreversible liver damage in this patient. This hypothesis can be further supported by a pathology study of the skin in patients with DENV infection, which showed endothelial swelling and extrusion of its plasma membrane into the capillary lumen, resulting in narrowing of the capillary lumen (*9*). Of note are the similarities between clinical findings in patients with DENV infection and sinusoidal obstruction syndrome such as hepatomegaly, ascites, right pleural effusion, swelling of the gall bladder wall, and decreased velocity or reversed direction of portal blood flow (*10*).

In conclusion, we report a case of liver failure from DENV infection with reversal of portal venous blood flow. We postulate that hepatic sinusoidal obstruction coupled with shock might be the underlying mechanism of liver failure in this disease.

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Publication of this case report was approved by the ethic committee, Faculty of Medicine, Chulalongkorn University.

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Spotted Fever Group Rickettsiae in Questing Ticks, Central Spain

To the Editor: The number of spotted fever group (SFG) rickettsiae that cause diseases in humans is rapidly increasing (*1*,*2*); infections have been described in ticks and humans in Spain (*3*,*4*). However, in Castilla-La Mancha, central Spain, where recreational parks and hunting estates are abundant and humans may be exposed to infected ticks, information on such infections is not available. Therefore, it is worthwhile to characterize *Rickettsia* spp. found in this area for epidemiologic studies and proper diagnosis of possible rickettsial diseases.

In this study, we obtained 148 questing adult ticks, representing the most abundant species in the area: 12 *Dermacentor marginatus*, 26 *Rhipicephalus bursa*, 41 *Rh. sanguineus*, 15 *Rh. turanicus*, 8 *Rh. pusillus,* 2 *Haemaphysalis punctata*, 11 *Hyalomma lusitanicum,* and 33 *Hyalomma marginatum* (*5*). The ticks were collected from the vegetation at natural sites surveyed in Castilla-La Mancha by blanket dragging with a cotton flannelette during fall 2009 and spring–summer 2010 (Figure, panel A) and classified (*5*).

Total DNA was extracted from dissected tick internal organs by using the DNeasy Blood & Tissue Kit (QIAGEN, Düsseldorf, Germany) and used to analyze *Rickettsia* spp. DNA by PCR, cloning, and sequence analysis of the amplicons. At least 3 clones were sequenced for each amplicon. Genes targeted by PCR included fragments of adenosine triphosphate synthase α subunit (*atpA*), heat-shock protein 70 (*dnaK*), outer membrane protein A (*ompA*), outer membrane protein B (*ompB*), citrate synthase (*gltA*), 16S rRNA, *recA*, and initiator protein of DNA replication (*dnaA*) (*6*,*7*). To characterize *Rickettsia* spp., we compared nucleotide sequence identity to reference strains and carried out multilocus analysis using *ompA-ompB* sequences and in silico *Pst*I and *Rsa*I restriction analysis of *ompA* sequences (*7*).

Ticks were first screened by 16S rRNA PCR, and positive samples were analyzed for all targeted genes. The results showed that 27 (18.2%) of the 148 ticks analyzed were positive for *Rickettsia* spp. Of these, 11 were confirmed as *R. massiliae* in *Rh. sanguineus*, *Rh. turanicus*, and *Rh. pusillus*, 3 as *R. raoultii* in *D. marginatus*, 2 as *R. slovaca* in *D. marginatus*, and 2 as *R. sibirica* subsp. *mongolitimonae* in *H. marginatum* and *Rh. pusillus* (Figure, panel B). These species had >99% pairwise nucleotide sequence identity to reference strains *R. massiliae* MTU5 (GenBank accession no. NC_009900), *R. slovaca* 13-B (accession no. NC_016639), and *R. sibirica* subsp. *mongolitimonae* HA-91 (accession no. AHZB00000000) genome sequences for all genes analyzed, and the only *R. raoultii* reported sequences (accession nos. JQ792107, JQ792166, JQ792134, and NR_043755 for *ompB*, *ompA*, *gltA*, and 16S rRNA, respectively). The sequences obtained in this study were deposited in the GenBank under accession nos. KC427998–KC428040.

Multilocus sequence analysis of *ompA-ompB* sequences (Figure, panel B) and in silico *Pst*I and *Rsa*I restriction analysis of *ompA* sequences also confirmed the identity of the *Rickettsia* spp. identified in this study. As previously shown (*7*,*8*), multilocus analysis with *ompA-ompB* sequences was highly informative about the

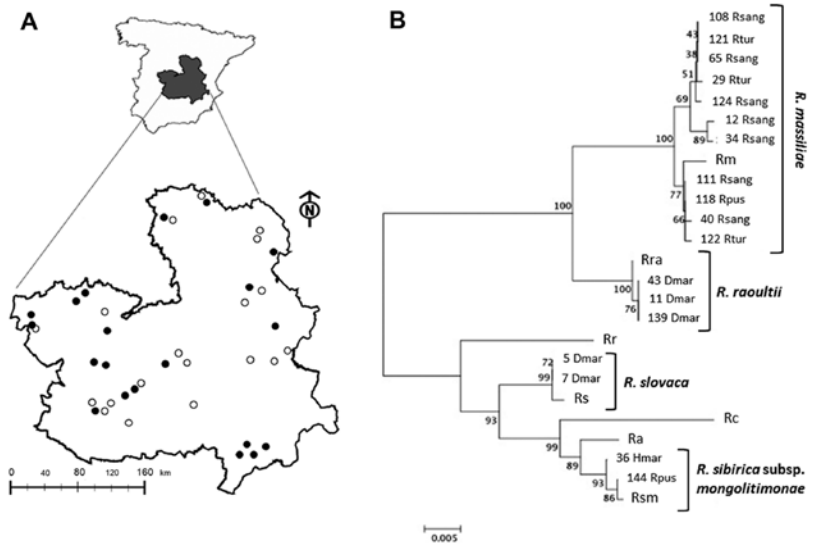

Figure. *Rickettsia* species in questing ticks collected in central Spain. A) Study area with 20 collection sites where ticks were found (black dots) of the 39 sites surveyed (white and black dots). B) Multilocus sequence analysis of *Rickettsia* spp. The evolutionary history was inferred by using the neighbor-joining method of *ompA-ompB* concatenated sequences (total length = 1,189 nt). The optimal tree with the sum of branch length = 0.15227017 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic relationship. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All ambiguous positions were removed for each sequence pair. Evolutionary analyses were conducted in MEGA5 (www.megasofware.net). The number of the *Rickettsia* spp. recognized in this study is shown next to the tick spp. Identified with them. Clusters of identified *Rickettsia* spp. are shown. Rc, *Rickettsia conorii* strain Malish 7; Ra, *R. africae* strain ESF-5; Rr, *R. rickettsii* strain Iowa; Rs, *R. slovaca* strain 13-B; Rm, *R. massiliae* strain MTU5; Rsm, *R. sibirica* subsp. *mongolitimonae* strain HA-91; *R. raoultii* isolate XG86; Rhsang, *Rhipicephalus sanguineus*; Rtur, *Rh. turanicus*; Rpus, *Rh. pusillus*; Dmar, *Dermacenter marginatus*; Hmar, *Hyalomma marginatum.* Scale bar indicates number of nucleotide changes per site.

phylogenetic relationship between *Rickettsia* spp. (Figure, panel B), with similar results for maximum likelihood, maximum parsimony, and neighbor-joining methods (data not shown). Furthermore, the results suggested the tick vectors for these *Rickettsia* spp. in the study area (Figure, panel B) match those reported or suspected previously for these *Rickettsia* spp. (*1*–*4*), but for the first time, *R. sibirica* subsp. *mongolitimonae* was identified in *Hyalomma* and *Rhipicephalus* spp. ticks in Spain (*4*).

These tick species are frequently found in the same area feeding on Eurasian wild boar (*Sus scrofa*) and red deer (*Cervus elaphus*), which may act as hosts for these pathogens (*5*,*9*). To test this hypothesis, we determined the seroprevalence for SFG rickettsiae in these host species in Castilla-La Mancha. Serum samples from 235 red deer and 206 wild boar were analyzed for the presence of anti-SFG *Rickettsia* antibodies by ELISA (Spotted Fever Rickettsia IgG EIA Antibody Kit, Fuller Laboratories, Fullerton, CA, USA). The ELISA was adapted to test ungulate serum specimens by substituting antihuman IgG-horseradish by protein G-horseradish peroxidase (Sigma-Aldrich, Madrid, Spain). Specific SFG-*Rickettsia* antibodies were detected in 146 (70.9%) of 206 wild boar and 174 (74.0%) of 235 red deer, indicating a high seroprevalence in these species and thus the possibility that they can serve as hosts for these pathogens.

These tick species also infest humans, thus posing a risk for transmission of rickettsiae that are pathogenic in humans (*1*). In fact, Castilla-La Mancha is one of the regions in Spain where a high number of SFG rickettsioses are reported ([*10*]; http://pagina.jccm.es/sanidad/salud/epidemiologia/3507.pdf).

In conclusion, these results demonstrate that SFG rickettsiae with public health relevance are found in ticks in central Spain as in other regions in Spain. In central Spain, the widespread distribution of tick vectors and possible wildlife hosts, the presence of persons in tick-infested recreational and hunting areas, and the transstadial and transovarial transmission of the pathogen in ticks may favor transmission to humans.

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Neonatal *Granulicatella elegans* Bacteremia, London, UK

To the Editor: *Granulicatella elegans*, a bacterium found in normal human oral flora, is generally associated with infective endocarditis. We discuss the identification and possible source of neonatal *G. elegans* bacteremia.

A 29-year-old woman sought care at Northwick Park Hospital (London, UK) at 41 weeks' gestation (first pregnancy) for spontaneous rupture of membranes and discharge of clear liquor. She had fever (37.6°C) and a heart rate of 98 beats/min; there was no evidence of fetal distress. The woman was released from the hospital.

Twelve hours later, she returned because of discharge of meconium-stained liquor. Her white cell count was 18 × 10^9/L (reference 3–10 × 10^9/L), and her C-reactive protein level was 277 mg/L (reference <5 mg/L). Emergency cesarean section was performed after a diagnosis of fetal distress. A large amount of foul-smelling meconium was observed. A live male infant (3.05 kg) was delivered; Apgar score was normal. Blood samples were cultured for suspected sepsis, and the neonate was empirically administered intravenous benzylpenicillin and amikacin (6 days). He made a full clinical recovery.

The mother remained generally well, although she had persistent tachycardia (120 beats/min) and fever (37.6°C). She was intravenously administered amoxicillin/clavulanic acid and amikacin; over the next 2 days, her white cell count became normal, but her C-reactive protein level remained >400 mg/L. By postdelivery day 10, her temperature and heart rate were normal. Antimicrobial drug treatment was stopped, and she was released without further treatment. We interviewed the mother 8 months later and established that she had no dental procedures/infection or endocarditis before, during, or after pregnancy.

Placental swab samples were cultured on Columbia horse blood agar (CBA) and chocolated CBA (both incubated aerobically with 5% CO_2 at 37°C for 24 hours), cysteine lactose electrolyte deficient agar (incubated in air at 37°C for 24 hours), and fastidious anaerobic agar with and without neomycin (incubated anaerobically at 37°C for 48 hours); all agar was from Thermo Fisher, Basingstoke, UK. On all media, the placental swab sample yielded moderate growth of tiny colonies, which Gram staining indicated were gram-positive coccobacilli.

Culture of the neonate's blood sample (BacTalert 3D; Becton Dickinson, Oxford, UK) grew small, gram-variable bacilli after 17 hours of aerobic incubation. A subculture incubated aerobically on CBA or chocolated CBA showed no bacterial growth; however, tiny colonies were seen on fastidious anaerobic agar with and without neomycin. Gram staining of the colonies showed gram-positive bacilli that were morphologically similar to those isolated from placenta. We suspected lactobacilli or streptococci, but testing (API Strep and Coryne strips; bioMérieux UK Ltd, Hampshire, UK) did not confirm this. Nutritionally variant streptococci were not suspected.

Both isolates were sent to the Health Protection Agency Laboratory of Healthcare Infections (London). Antimicrobial drug sensitivities were determined (Iso-Sensitest Agar; Thermo Fisher) according to a previously defined method (http://bsac.org.uk/wp-content/uploads/2012/02/Version-11-2012-Final-.pdf).The isolates were sensitive to penicillin, clarithromycin, trimethoprim, and vancomycin but resistant to tetracycline. Partial sequencing of the 16S rRNA genes of both isolates confirmed they were the same *G. elegans* strain. Isolates exhibited enhanced growth in the presence of pyridoxal (Figure) satellitism with *Staphylococcus aureus*.

G. elegans (originally known as *Abiotrophia elegans*) was first described in 1998 (*1*) as a catalase-negative, oxidase-negative, nonmotile, facultative anaerobic gram-positive bacterium. However, the bacterium can exhibit variability and pleomorphism on Gram staining: forms range from bacilli in nutrient-depleted media to cocci arranged in short chains in nutrient-rich media (*1*,*2*). This variability poses challenges to the identification and taxonomic classification of the organism. The possibility of nutritionally variant streptococci (NVS) should be considered when gram-positive cocci or bacilli are seen on Gram staining, but the cocci/bacilli grow poorly on non-supplemented media. Studies have shown that pyridoxine facilitates the growth of NVS (*3*).

Antibiotic susceptibility tests for NVS should be performed on media supplemented with pyridoxal (*3*). NVS are usually susceptible or moderately susceptible to penicillin. Strains tolerant to penicillin have also been reported, especially in the presence of supplements (e.g., pyridoxal and cysteine). High-dose penicillin and aminoglycoside are recommended for the treatment of serious NVS infections (*3*).

G. elegans bacteria can be part of normal oral flora (*4*); however, the bacteria are predominantly isolated from blood cultures for patients with infective endocarditis. Gonzales-Marin et al. (*5*) detected *G. elegans* in nasogastric isolates of neonates. The authors concluded that hematogenous translocation of maternal oral flora into the amniotic environment was the likely source because, the authors stated, *G. elegans* is not part of the normal vaginal flora. However, *G. elegans* has been isolated from the vaginal tract of healthy women (*6*).

Early-onset neonatal bacteremia caused by *G. adiacens* has also been reported (*7*). Molecular studies identified the same organism in the maternal cervical flora, suggesting ascending infection or acquisition of the bacteria by the neonate during delivery.

We cannot be certain whether the neonatal *G. elegans* bacteremia was caused by ascending or transplacental infection. We think the former is more likely in the light of the relatively prolonged rupture of membranes and the presence of chorioamnionitis.

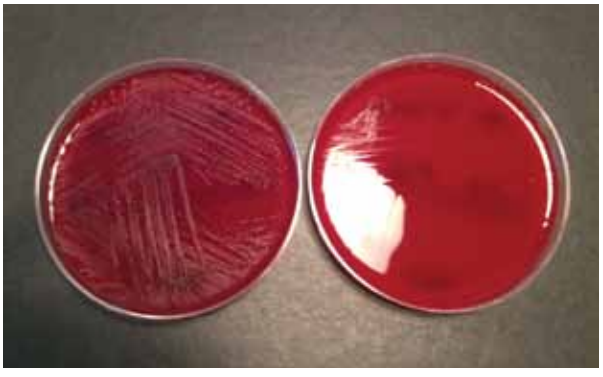

Figure. Blood agar plates with (left) and without (right) pyridoxal supplement from a study of neonatal Granulicatella elegans bacteremia, London, UK.

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Bartonella Species in Raccoons and Feral Cats, Georgia, USA

To the Editor: *Bartonella* spp. are vector-borne, facultative, intracellular bacteria that infect mammalian erythrocytes and endothelial cells and might cause chronic bacteremia and asymptomatic infections in reservoir hosts (*1*). There are currently 30–40 identified *Bartonella* species (*2*), and 14 of them are zoonotic; they have a wide variety of reservoirs, including rodents, carnivores, and ungulates (*3*). This study describes 2 *Bartonella* species in an urban population of raccoons and compares these findings to *Bartonella* infection in sympatric feral cats (*Felis catus*).

Raccoons (*Procyon lotor*) (n = 37) were live-trapped (Tomahawk Life Trap Company, Tomahawk, WI, USA) in spring and summer of 2012 on St. Simons Island, an urbanized coastal barrier island in Georgia in the southeastern United States (31°9′40″N, 81°23′13″W). The island is characterized by beach, salt marsh, forest, freshwater slough, and extensive residential developments. Raccoons were anesthetized with 20 mg/kg ketamine (Aveco Co., Fort Dodge, IA, USA) and 4 mg/kg xylazine (Mobay Corp., Shawnee, KS, USA), and blood was collected from the jugular vein into tubes containing EDTA. Feral cat blood samples (n = 37) from trap-neuter programs were collected by local veterinarians on St. Simons Island. Institutional Animal Care and Use Committee (A2011 03-042-Y2-A2) and Georgia Department of Natural Resources wildlife permits (29-WBH-12-100) were obtained before sampling.

DNA was extracted from blood by using a commercial DNA extraction kit (Quick-gDNA MiniPrep; Zymo Research Corp., Orange, CA, USA). Extracted DNA was used to amplify the 16S–23S rRNA intergenic spacer region of *Bartonella* spp. by nested PCR. For outer PCR, we used primers QHVE-1 (5′-TTCAGATGATGATCCCAAGC-3′) and QHVE-3 (5′-AACATGTCTGAATATATCTTC-3′) (*4*,*5*). PCR was performed with an initial incubation for 2 min at 94°C; 35 cycles of denaturation at 94°C for 30 s, primer annealing at 52°C for 30 s, and elongation at 72°C for 60 s; and a final incubation at 72°C for 6 min.

Nested PCR was performed by using primers QHVE-12 (5′-CCG GAG GGC TTG TAG CTC AG-3′) and QHVE-14b (5′-CCT CACAAT TTC AAT AGA AC-3′) (*4*). Nested PCR conditions were identical to those for the outer PCR, except for the annealing temperature, which was 55°C. Positive amplicons were separated by electrophoresis on a 1.2% agarose gel and purified by using the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA, USA).

Purified DNA amplicons (400–600 bp) were sequenced by using an ABI automated sequencer (Applied Biosystems, Foster City, CA, USA). Intergenic spacer sequences from raccoon isolates were aligned with reported *Bartonella* species sequences in GenBank by using the ClustalW algorithm (*6*). A phylogenetic tree of the sequences was constructed by using neighbor-joining methods and maximum composite likelihood distances. Data were resampled 1,000 times to generate bootstrap values by using MEGA5 (*7*).

Of 74 samples analyzed (37 raccoon, 37 feral cat), 16 (43%) raccoon samples and 18 (48%) feral cat samples were positive for *Bartonella* spp. by PCR. Thirteen positive raccoon samples and 16 positive feral cat samples were sequenced. Twelve positive raccoon samples and 13 positive feral cat samples contained *Bartonella henselae. B. koehlerae* was amplified from 1 feral cat sample and 1 raccoon sample (99% sequence homology with a *B. koehlerae* sequence, GenBank accession no. AF312490). Two feral cat samples were identified as containing *B. clarridgeiae* and showed 98% and 100% sequence homology with a *B. clarridgeiae* sequence (GenBank accession no. AF167989) (Table; Figure, Appendix, wwwnc.cdc.gov/EID/article/19/7/13-0010-F1.htm).

This study identified *B. henselae and B. koehlerae* in feral cat and raccoons and *B. clarridgeiae* in feral cats. Our results are useful because raccoons are potential reservoir hosts of zoonotic *B. henselae* and *B. koehlerae*, in addition to *B. rochalimae*, and there could be cross-species transmission of *Bartonella* spp. between feral cats and raccoons.

Among reservoir hosts for *Bartonella* species, rodents and cats have been the most extensively studied. Rodents harbor 11 *Bartonella* species (*3*). Cats are the principal reservoirs of *B. clarridgeiae,* which causes endocarditis in humans, and *B. henselae*, which causes cat-scratch disease. However, little is known about *Bartonella* spp. infections in raccoons; there is only 1 report of *B. rochalimae* in raccoons in California (*8*).

In this study, a relatively high proportion of raccoons were infected with *B. henselae*, implying that there is spillover of *B. henselae* from feral cats to raccoons or that raccoons are another active reservoir for *B. henselae*. *B. clarridgeiae* and *B. koehlerae* are also

Table. *Bartonella spp.*–positive raccoons and feral cats identified by PCR and sequencing of DNA extracted from whole blood, Georgia, USA*

Bartonella species	Raccoon (*Procyon lotor*)	Feral cat (*Felis catus*)
B. henselae	12/37	13/37
B. koehlerae	1/37	1/37
B. clarridgeiae	0/37	2/37

*Values are no. positive/no. tested.

zoonotic; cats are primary reservoirs, and humans and dogs are accidental hosts (*1*). However, *B. clarridgeiae* was recently detected in rodent fleas in China (*9*) and *B. koehlerae* was isolated from feral pigs from the southeastern United States (*10*), suggesting that these pathogens also have multiple reservoir species.

Clarifying whether *Bartonella* infections in raccoons are caused by spillover from feral cats needs further study. Additional samples from raccoons and other species in urbanized and undeveloped habitats with different host species composition (e.g., cat-free environment) might enable further *Bartonella* spp. characterization in wildlife. We suspect urban raccoons and feral cats play a major role in *Bartonella* spp. transmission.

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Rifampin-Resistant *Mycobacterium bovis* BCG–Induced Disease in HIV-Infected Infant, Vietnam

To the Editor: Guidelines for the diagnosis and management of *Mycobacterium bovis* BCG disease in HIV-infected children are lacking. BCG strains are intrinsically resistant to pyrazinamide and in some cases have low-level resistance to isoniazid (*6*). However, data on acquired drug resistance in *M. bovis* BCG are limited. We describe a case of BCG disease caused by a rifampin-resistant strain of *M. bovis* BCG in an HIV-infected infant in Vietnam.

The daughter of a known HIV-infected woman, who did not fully adhere to antiretroviral therapy (ART) during pregnancy, received the *M. bovis* intradermal BCG (Pasteur strain) vaccine at birth. HIV infection was diagnosed in the infant by PCR when she was 8 weeks of age. At 9 months of age, she was admitted to the Pediatric Infectious Diseases Department of the Pham Ngoc Thach Hospital (Ho Chi Minh City, Vietnam) because of a voluminous ipsilateral axillary mass at the site of the vaccination, fever, weight loss, and hepatosplenomegaly. The percentage of CD4+ T cells was 27% (1,620 cells/mm^3). Regional BCG disease was clinically diagnosed without microbiological investigation, and a broad antimycobacterial therapy targeting *M. tuberculosis* complex species was started with 5 mg/kg isoniazid, 10 mg/kg rifampin, and 25 mg/kg pyrazinamide. After 6 weeks of antimycobacterial therapy, ART was initiated with lamivudine, stavudine, and abacavir.

After 6 months of antimycobacterial treatment, the infant was hospitalized again for recurrent inflammation and fistulization of the axillary lymph nodes associated with fever.

Fluid from the axillary mass was collected by fine-needle aspiration for bacteriologic investigations. Direct microscopic examination showed acid-fast bacilli, and the mycobacterial infection was confirmed by culture. By using conventional biochemical methods, the mycobacterial isolate was assigned to the *M. bovis* species. Pyrazinamide was discontinued, and antimycobacterial therapy was continued for 4 supplementary months with rifampin (15 mg/kg) and isoniazid (10 mg/kg). After 2 months, drug susceptibility testing results confirmed pyrazinamide intrinsic resistance and isoniazid and ethambutol susceptibility and showed rifampin resistance. The late inflammatory reaction after introduction of ART was evocative of immune reconstitution inflammatory syndrome. Nevertheless, drug resistance may have contributed. Despite the rifampin resistance, the patient showed clinical improvement, and the rifampin/isoniazid treatment was continued for 2 more months. The child's BCG disease was cured on completion of 10 months of antituberculous treatment.

Retrospective molecular investigations using the GenoType MTBC Kit (Hain Lifescience, Nehren, Germany) enabled identification of the isolate stored at −80°C as *M. bovis* BCG strain. A mutation in the *rpoB* gene (codon 531, Ser531Tyr) associated with rifampin resistance was detected by using the GenoType MTBDRplus Kit (Hain Lifescience) and partial sequencing of the *rpoB* gene (*5*,*7*). No mutation in the *katG* and *inhA* genes, frequently associated with isoniazid resistance, was detected.

To our knowledge, this case is the second report of rifampin-resistant *M. bovis* BCG disease in HIV-infected children. The first report involved a child in South Africa who was vaccinated with the Danish BCG strain (*4*); this strain shows low-level resistance to isoniazid and therefore has a high risk of evolving to multidrug resistance in instances of suboptimal isoniazid levels. The *M. bovis* BCG Pasteur strain (American Type Culture Collection 35734) used for vaccination in Vietnam is isoniazid and rifampin susceptible and pyrazinamide resistant (*9*). Despite appropriate antimycobacterial treatment, the relatively low doses of isoniazid (5 mg/kg), poor adherence, or inadequate absorption of drugs because of HIV-related gastrointestinal disease may have resulted in subtherapeutic in vivo drug concentrations and thus in selection of a drug-resistant *M. bovis* BCG strain. This case should alert clinicians of the possible emergence of rifampin resistant *M. bovis* BCG strains.

Because disseminated BCG disease in HIV-infected children presents a high risk for illness and/or death, these patients should receive optimal tuberculosis treatment (*2*) based on 4-drug (rifampin, isoniazid, ethambutol, and pyrazinamide) regimen doses for at least 9 months until *M. tuberculosis* is ruled out (*3*). Untreated local BCG immune reconstitution inflammatory syndrome may not necessarily progress to dissemination; therefore, treatment would not appear necessary (*8*).

Some studies suggest that the survival of HIV-infected children with BCG disease could be attributed to early initiation of ART in combination with other treatments (*1*,*3*). In the South Africa case, the child died, and the authors suggested that this outcome was related to the severity of the clinical features, the severe HIV-related immune suppression, and the absence of ART (*4*). In the case in Vietnam, despite the emergence of drug resistance, the early initiation of ART in a child with a localized disease, the persistent efficacy of isoniazid, and the spontaneous fistulization of the abscess probably contributed to the good outcome for the infant.

In conclusion, this case highlights the challenges in management of BCG disease in children. It also emphasizes the possible risk for emergence of acquired drug resistance in *M. bovis* BCG strains, complicating the medical management of such cases.

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Bulleidia extructa Periprosthetic Hip Joint Infection, United States

To the Editor: *Bulleidia extructa* is an obligately anaerobic, nonmotile, non–spore-forming gram-positive bacillus first described in 2000 by Downes et al. (*1*), after having isolated a bacterium from the oral cavity of persons with periodontitis and dentoalveolar abscesses that did not correspond to any known species. After phenotypic and genetic characterization, the investigators proposed a new genus, *Bulleidia*, and the species *B. extructa*. Since then, additional reports have associated the organism with oral infections, specifically periodontal disease (*2–5*). While *B. extructa*'s association with human periodontal disease is well documented, the bacterium has so far not been implicated in other pathogenic processes. We report here a case of a total hip arthroplasty infection caused by *B. extructa* in an immunocompetent patient.

In November 2010, an 82-year-old man with a non-cemented right total hip arthroplasty that was performed 26 years previously was evaluated for right hip pain. He had been in his usual state of health without any complaints until a month earlier, when he lost his footing and hyperabducted his hip joints, involuntarily performing a split, while washing a boat cover with a power washer. Since then, he reported right hip pain that somewhat limited his mobility.

Physical examination revealed an antalgic gait, mild swelling of the right lower extremity, and impaired hip mobility related to pain on the right side, specifically with extension, flexion, abduction, and adduction. Results of the patient's blood work were notable for normocytic anemia (hemoglobin 10.6 g/dL), thrombocytosis (459 × 10^9/L), elevated erythrocyte sedimentation rate (101 mm/h), and elevated C-reactive protein (88.7 mg/L). Leukocyte count was within normal limits (9.6 × 10^9 cells/L). An ultrasound examination of the right hip joint showed extensive synovitis and a large, 4.3 × 5.0 × 5.1–cm vascular mass extending anteriorly from the joint space. Aspiration of the joint space yielded 1 mL of blood-stained fluid with 111,595 cells/μL (95% neutrophils, 5% monocytes/macrophages). Anaerobic bacterial culture grew a gram-positive bacillus identified as *B. extructa* by partial 16S rRNA sequencing. DNA was prepared for PCR amplification by using PrepMan Ultra (Applied Biosystems, Foster City, CA, USA) and amplified and bidirectionally sequenced by using primers 5′-TGGAGAGTTTGATCCTGGCTCAG-3′ and 5′-TACCGCGGCTGCTGGCAC-3′. The generated 484-bp sequence differed by 2 bp from 483 bp of available sequence from *B. extructa* GenBank accession no. AF220064. The isolate was susceptible to penicillin, clindamycin, and metronidazole by using E-test.

The patient underwent total hip arthroplasty resection. Intraoperatively, purulence was noted upon entering the hip joint. Histopathologic examination of removed tissue revealed acute inflammation. Five hip tissue specimens were obtained for culture; 3 specimens yielded *B. extructa*. Six weeks of intravenous ceftriaxone treatment was prescribed, and the patient was instructed to revisit a dentist for a full dental examination. Before seeking treatment for this episode, he reported that he was seeing a dentist on a regular basis and denied any recent dental surgery or infections.

The patient was seen in a follow-up visit 2 months after reimplantation surgery; at that time, he reported minimal pain and had begun to bear weight on the affected side. There was no evidence for infection recurrence.

Periprosthetic joint infections are a major complication after joint replacement. The number of procedures for total hip and knee replacements has

increased during the past 13 years (*6*). This trend is accompanied by an increase in the total number of periprosthetic joint infections, even though the overall percentage of this complication is low (*7*). The most commonly isolated organisms in periprosthetic joint infections are gram-positive cocci, specifically *Staphylococcus aureus* and *S. epidermidis* (*8*). In a retrospective review, Moran et al. (*9*) examined the microbiological spectrum of 112 patients undergoing debridement and irrigation for a periprosthetic joint infection (hip [52], knee [51], elbow [4], ankle [3], shoulder [2]) at a tertiary care center in the United Kingdom during 1998–2003. The most frequently isolated microorganisms were coagulase-negative staphylococci (47%) followed by methicillin-sensitive *S. aureus* (44%), methicillin-resistant *S. aureus* (8%), aerobic gram-negative organisms (8%), and anaerobes (7%). Thirty-seven percent of patient specimens grew multiple microorganisms.

We document the ability of *B. extructa* to cause an infection beyond its usual habitat, the oral flora. We hypothesize that the infection in this patient might have developed from hematogenous seeding in which an undiscovered and asymptomatic oral infectious nidus might have served as the seeding focus while mild trauma to the hip could have facilitated access to the joint space.

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Tick-borne Encephalitis Virus, Zealand, Denmark, 2011

To the Editor: In Scandinavia, the incidence of tick-borne encephalitis (TBE) is increasing and expanding its geographic range (*1*). TBE virus (TBEV) types TBEV-Eur and TBEV-Sib occur in Estonia and Finland, along with 2 tick species, *Ixodes persulcatus* and *I. ricinus*. In Denmark, TBE has been reported since the 1950s only from the isolated Bornholm Island in the Baltic Sea with an incidence of ≈4 cases per 100,000 persons (*2*). Statistical climate-matching models based on the known spatial distribution of TBEV indicate that the present North Zealand climate also would support TBEV-Eur transmission cycles (*3*). Recently (2008 and 2009), we reported TBE in 2 persons who had histories of tick-bite and originated from a single location in a small forest area (Tokkekøb Hegn) in North Zealand where TBE was previously unrecognized (*4*).

To determine whether TBE was established in this possibly new TBE focus in mainland Denmark outside Bornholm, we collected ticks by flagging (*4*) from 3 sites at Tokkekøb during June–July 2011. The 3 sites yielded 896 ticks (854 nymphs, 22 male adults, 20 female adults) in 24 pools. A fourth site at Grib Forest 10 km to the north yielded 198 ticks (183 nymphs, 9 male adult, 6 female adults) in 13 pools.

Flagging was repeated in September 2011 at Tokkekøb to confirm the presence of TBEV and to obtain material suitable for virus isolation. Here, we obtained 7 pools (100 nymphs each) and 1 pool with adults (15 male, 15 female). In September 2011, we also obtained 13 pools (738 nymphs, 37 male adults, 41 female adults) at 3 suspected TBE locations on Bornholm Island. In addition, 1,073 ticks in 58 pools were collected in 2010 and 2011 from deer inspected by the National Center for Wildlife Health from 54 various locations (Figure, panel A). All ticks were identified as *I. ricinus* on the basis of morphology. For TBEV-specific real-time PCR (*5*), ticks were homogenized in 0.5 mL nucleic acid extraction buffer and RNA/DNA extracted from 0.2 mL homogenate by using the MagNA Pure total NA kit (Roche, Indianapolis, IN, USA). Three of 37 pools (2 with nymphs, 1 with adult females) from Tokkekøb were TBEV RNA positive. None of 58 tick pools from other locations in Denmark or Bornholm were positive for TBEV but contained other pathogens (*6*). Five of the 8 pools obtained from the second flagging session (all nymphs) in Tokkekøb were TBEV PCR positive, and 2 yielded isolates (T2, T3) in VeroB4 cell culture. Considering that the duration of the nymphal stage in *I. ricinus* is usually only 1 or 2 years in northern Europe (*7*), the repeated identification of TBEV in nymphs at the same location in 2009 and 2011 indicates establishment of a new focus of endemic TBEV in Denmark.

Phylogenetic analysis of TBEV-E sequences (1,488 nt) of central European (*8*) and Scandinavian TBEV strains did not group the Zealand isolate T2 (T3 was not sequenced) with the Bornholm strain but into a subclade with 2 isolates from Sweden, Torö-2003 (*9*) (GenBank accession no. DQ401140) and Saringe-2009 (GenBank accession no. KC469073); an isolate from Norway (GenBank accession no. EF565947), and isolates from North Bohemia (Czech Republic). The Bornholm strain located into a different subclade containing various sequences from South and Central Bohemia (Figure, panel B). TBEV sequences from the Baltics and Finland locate to a spate clade. The missing link between the isolates from Bornholm and Zealand also was observed in a median joining network analysis (Splits Tree program, Epsilon1 [www.splitstree.org], 2,000 iterations [data not shown]).

Two severe clinical cases of TBE connected to this new focus occurred in 2008–2009 (*4*). To search for additional missed clinical TBE cases from this area, we examined serum and cerbrospinal fluid of 96 patients (2007–2009) in whom encephalitis developed after tick bite; these samples were found negative for *Borrelia* spp. by antibody ELISA and PCR (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/7/13-0092-Techapp1.pdf). To assess anti-TBEV seroprevalence, we also tested serum from 78 patients experiencing "summer flu" who had histories of tick bite; this serum was submitted by general practitioners in North Zealand during July–November 2010 (online Technical Appendix). Except for 1 patient infected in Bornholm and 2 patients infected in Sweden, none were

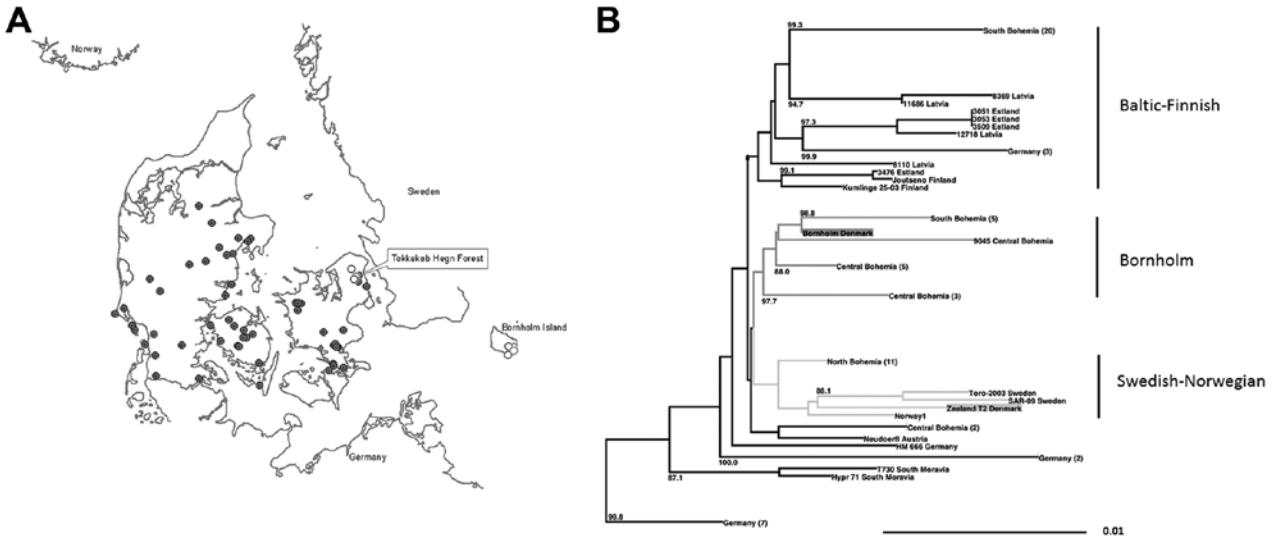

Figure. A) Tick collection areas in Denmark. Red indicates ticks sampled from animals; blue indicates flagging. B) Neighbor-joining phylogenetic analysis of a 1,488-nt set of 78 tick-borne encephalitis virus (TBEV)–Eur E gene sequences including reference strains Neudoerfl (Austria) and Hypr 71 (South Moravia) performed in ClustalW with a 1,000 bootstrap approach (LASERGENE, MEGALIGN, DENDROSCOPE) outgrouped to Louping ill virus (data not shown). Sequence designations of central European strains as in (*8*). Dark gray indicates Denmark Bornholm clade; white indicates Swedish-Norwegian clade. For simplicity, some subtrees were collapsed; these are designated with region and number of sequences in the collapsed subtree in brackets.

positive by ELISA (Enzygnost Anti-TBE/FSME Virus [IgG, IgM] Siemens, Erlangen, Germany) or PCR (online Technical Appendix). Since the 1980s, Sweden has experienced a 4-fold increase in human TBE incidence, with spread southwest (*10*). The emergence of the TBEV strain T2 closely related to isolates from Sweden may be a continuation of this geographic trend. A previous antibody study found 3 deer positive for TBEV in Zealand-Falster (*2*); however, without convincing neutralization data, this finding is not confirmed. The lack of TBEV viremia and seropositivity among the patients in Zealand who had histories of tick bites supports a recent introduction to the new focus. Thus, 2 distinct introductions of TBEV have occurred in Denmark. The underlining environmental or climatic factors driving this geographic trend remain unknown.

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Usutu Virus in Migratory Song Thrushes, Spain

To the Editor: Usutu virus (USUV), a member of the Japanese encephalitis virus antigenic group, was first detected in 1959 in mosquitoes in South Africa (*1*), and it emerged in 1996 in blackbirds (*Turdus merula*) in Italy (*2*). Recent cases of USUV infection in asymptomatic blood donors (*3*) and severe disease in immunocompromised persons (*4*) have shown its zoonotic potential.

Epidemiology and molecular phylogeny of USUV isolated in Italy, Austria, Hungary, Switzerland, and Germany suggest that stable endemic mosquito–bird cycles have been established in Europe (*5*,*6*). Where active vector surveillance programs exist, USUV is detected in mosquitoes before bird deaths and human infections. USUV strains similar to African strains were detected in mosquitoes in Spain in 2006 and 2009 (*7*,*8*).

In November 2012, two live song thrushes (*Turdus philomelos*) with central nervous system signs were recovered from a die-off of ≈10 birds at a hunting estate in southern Spain. A full necropsy was conducted on the 2 thrushes (which died shortly after capture), and samples were collected for virus detection and histopathologic examination. Total RNA was extracted from oral and cloacal swab specimens, from serum from a cardiac blood clot, and from heart, kidney, spleen, and brain tissues by using High Pure RNA Tissue Kit (Roche Diagnostics, Barcelona, Spain) and analyzed by generic flavivirus SYBR Green (QIAGEN, Madrid, Spain) real-time reverse transcription PCR (RT-PCR) and by a generic conventional nested flavivirus RT-PCR (*9*). The product of the first PCR (1,048 bp) was resin purified, cloned into pGEM-T (Promega, WI, USA), and sequenced.

This sequence was compared with sequences of European and African USUV strains that were available in GenBank. In addition to the histopathologic examination, we used a polyclonal primary rabbit antibody directed against West Nile virus with proven cross-reactivity to other flaviviruses for viral antigen detection by immunohistochemical testing (*9*).

The thrushes were an adult male and female in poor body condition; they had greenish urate-soiled feathers around the cloaca. Subcutaneous or visceral fat deposits were absent, and the pectoral muscle was partially atrophied, more severely in the male. Both birds showed severe generalized congestion.

The serum, brain, and pool of cloacal and oropharyngeal swab specimens of both birds yielded a strongly positive signal in the generic flavivirus real time RT-PCR. Sequencing of the PCR product obtained in the generic flavivirus RT-PCR (GenBank accession no. KC437386) showed a 96%–97% homology to published USUV sequences. Nucleotide sequence analysis revealed a higher homology to Northern European strains (97% to BH65/11-02-03 [HE599647] and Meise H, Germany [JQ219843]; Budapest, Hungary [EF206350]; Italy 2009 [JF266698]; and Vienna 2001, Austria [AY453411]) than to a USUV strain isolated in South Africa (96% to SAAR-1776 [AY453412]). This finding was also supported by phylogenetic analysis of USUV strains (Figure, panel A), with similar results for maximum likelihood and neighbor-joining methods (data not shown).

Histologically, both birds had severe encephalitis characterized by congestion, neuronal and Purkinje cell necrosis, gliosis, satellitosis, neuronophagia, and endothelial cell swelling and vasculitis. Other lesions included multiorgan congestion, necrosis of renal tubular epithelium, and moderate hemosiderosis in the liver and spleen. Intravascular

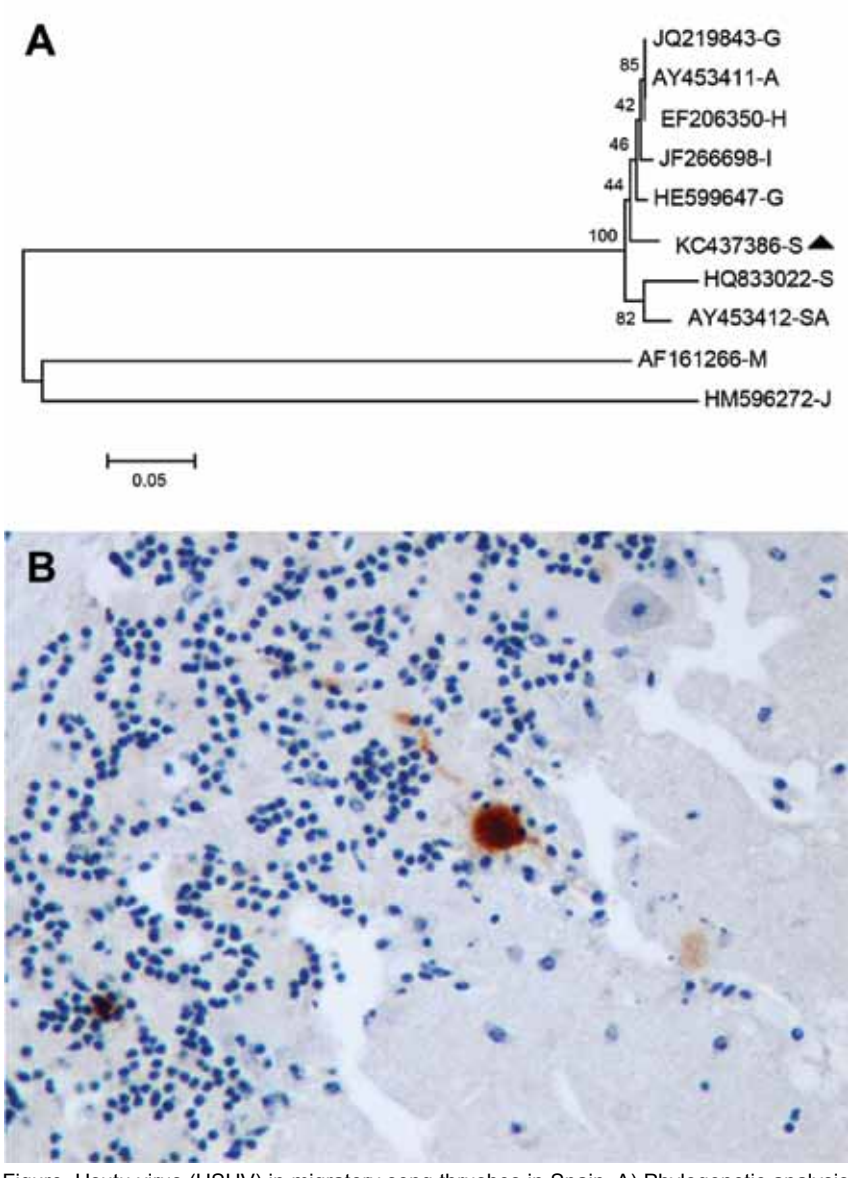

Figure. Usutu virus (USUV) in migratory song thrushes in Spain. A) Phylogenetic analysis of European and African USUV strains. The evolutionary history was inferred using the neighbor-Joining method. The optimal tree with the sum of branch length = 1.18014408 is shown. The percentages of replicate trees 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 by using the maximum composite likelihood method and are in the units of the number of base substitutions per site. The analysis involved 10-nt sequences. All ambiguous positions were removed for each sequence pair. There were a total of 929 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (www.megasoftware.net). USUV strains are identified by their GenBank accession numbers and a capital letter indicating the country of origin (A, Austria; G, Germany; I, Italy; H, Hungary; S, Spain; SA, South Africa); the sequences of the last 2 branches correspond to outgroup viruses used to root the tree (M, Murray Valley encephalitis virus [GenBank AF161266]; J, Japanese encephalitis virus [GenBank HM596272]). The song thrush strain from Spain 2012 is highlighted with a black triangle. Scale bar indicates nucleotide changes per site. B) Immunohistochemical staining with cross-reacting antibody showing USUV antigen labeling in a Purkinje cell of the cerebellum of a song thrush that died from encephalitis. Original magnification x400.

cross-sections of filarial parasites were detected in pulmonary capillaries. Antigen labeling was detected in neurons in the cerebral hemispheres and brain stem and in glia cells throughout the brain (Figure, panel B). Rare Purkinje cells and neurons in peripheral ganglia (e.g., of the gizzard) as well as cardiac myofibers and renal tubular epithelial cells were positive for viral antigen.

The molecular genetic analysis, histopathologic examination, and immunohistochemical testing confirmed encephalitis caused by USUV in 2 migratory wintering song thrushes who died during a small mortality event in southern Spain. Phylogenetic analysis showed that the USUV strain infecting the diseased birds was more similar to USUV strains from Austria, Hungary, and Germany than to USUV strains isolated from mosquitoes in Spain (*7*,*8*) or on the African continent. This result, together with the fact that song thrushes are a nonresident, migratory, wintering bird species in southern Spain (*10*), provides circumstantial evidence of USUV introduction into Spain during bird migration from northern Europe. Persistence of USUV in song thrushes and recrudescence of the infection during southward migration with concomitant filarial infection, together with the presence of USUV in a local endemic cycle from previous introductions, high vector abundance, and high viral loads in infectious mosquitoes, are possible scenarios that caused this outbreak.

Our data imply that introduction of USUV (and potentially other flaviviruses such as West Nile virus lineage 2, which has not yet been detected in Spain) from Northern Europe, in addition to local endemicity and introduction from Africa, occurs, and that the zoonotic European USUV strain may be co-circulating with strains of African origin. At this time, it is not clear whether USUV strains of Spanish/African lineage differ in virulence for humans from strains from the European/African lineage. However, virus introduction by northern migrants, in combination with locally favorable conditions for vector populations, implies a risk for virus amplification and transmission and disease outbreaks in humans and horses outside the currently established mosquito-trapping period (May–October) of targeted flavivirus surveillance programs.

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Rickettsia aeschlimannii Infection in a Man, Greece

To the Editor: In Greece, 6 spotted fever group (SFG) *Rickettsia* species have been detected in ticks: *Rickettsia conorii, R. massiliae, R. aeschlimannii*, *R. sibirica mongolitimonae*, *R. slovaca*, and *R. rhipicephali* (*1*). SFG species present characteristic clinical signs, including high fever, headache, and maculopapular rash; an inoculation eschar at the tick bite site is characteristic of some, but not all, SFG rickettsioses. Symptoms during the early stages of illness are nonspecific, and diagnosis is a challenge for physicians who are not familiar with rickettsial diseases. So far, 2 SFG *Rickettsia* species have been implicated in human disease in Greece: Mediterranean spotted fever caused by *R. conorii* (*2*), and lymphagitis-associated rickettsiosis (LAR) caused by *R. sibirica mongolitimonae* (*3*). We report a rickettsiosis case in a man on the island of Crete, Greece caused by a third *Rickettsia* species belonging to the SFG, *R. aeschlimannii*.

During June 2010, a 70-year-old man residing in an agricultural area of eastern Crete was admitted to the emergency unit of General Hospital of Agios Nikolaos for evaluation of a reddish, painless papule on the anterior surface of his left arm. The papule was 2 cm in diameter, and was surrounded by a less reddened infiltrated area 8 cm in diameter (Figure). The area was without tenderness or pruritus. At the center of the papule, which was cyanotic, the presence of a tick was recorded, and the tick was removed carefully in its entirety. The patient was afebrile and reported no other symptoms. The papule had developed within few hours, although 5 days previously, the patient had noticed a dark colored nodule on his left arm but paid no attention to it. The patient reported that rabbits were bred and goats and sheep grazed at close proximity to his residence.

Serum and whole blood samples were drawn, and a local skin biopsy was performed from the center of the skin lesion. Laboratory tests revealed a high level of C-reactive protein, microscopic hematuria, and a leukocyte count of 6.01×10^9 cells/L. Hepatic enzymes alanine transaminase and aspartate aminotransferase were within normal ranges. The patient was treated with doxycycline, 100 mg twice daily for 7 days; he did not develop further symptoms, and the skin lesion healed without ulceration.

All samples were sent to the Laboratory of Clinical Bacteriology, Parasitology, Zoonoses and Geographic Medicine at the University of Crete for further testing. The tick was identified as *Rhipicephalus turanicus* by using standard taxonomic keys (*4*). IgG and IgM titers reactive to SFG rickettsiae antigens were determined by an immunofluorescence antibody assay as described by the manufacturer (bioMérieux, Marcy l'Etoile, France).

Twenty days after initial assessment and treatment, convalescent-phase blood samples were drawn for serum and whole blood analysis. Titers against *R. conorii* were detected in both the initial samples (IgM 1/100, IgG 1/60) and the convalescent-phase samples (IgM 1/100, IgG 1/120). DNA was extracted from the blood samples, the skin biopsy, and the tick by using a QIAamp Tissue Kit (QIAGEN, Courtaboeuf, France) and used as a template in previously described PCR assays by using primers RpCS 877p-RpCS 1258n and Rr19070p-Rr190602n, targeting a 381-bp portion of the *gltA* and a 532-bp portion of the *ompA* genes of *Rickettsia* spp. (*5*). The whole blood drawn in the hospital, the skin biopsy, and the tick were positive for both genes. However, the convalescent-phase blood sample was negative.

PCR products were purified by using the QIAquick Spin PCR Purification Kit (QIAGEN) and sequenced (Bioanalytica–Genotype, Athens, Greece) according to the manufacturer's instructions. Sequences obtained shared 100% similarity to the corresponding fragment of the genome of *R. eschlimannii* (*gltA*: JF803904;

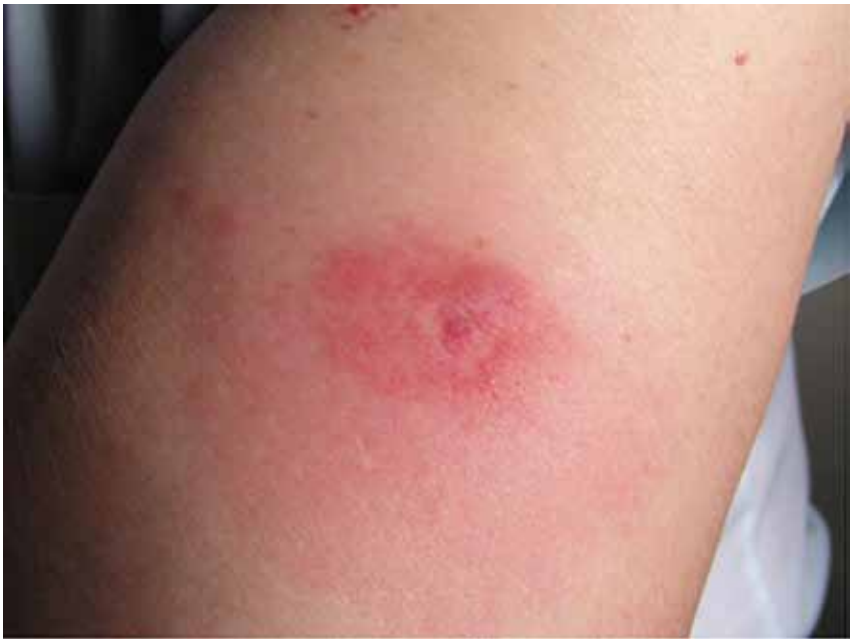

Figure. Papule on the anterior surface of the left arm of a 70-year-old man, Crete, Greece. The papule was surrounded by an infiltrated area without tenderness or pruritus. A tick was found in the center of the papule and carefully removed in its entirety.

ompA: JF803906). All samples were cultured in human embryonic lung fibroblasts as described (*6*). After 4 weeks, no bacteria were isolated.

We report a human case of *R. eschlimannii* infection in Crete, Greece. Our finding was confirmed by molecular methods. However, we were not able to cultivate *R. aeschlimannii* from samples collected. This result suggests that living microorganisms may have died before testing or that only DNA, but no living organism, was present in the samples. *R. aeschlimannii* was first isolated from *Hyalomma marginatum* ticks from Morocco (*7*). In Europe, *R. aeschlimannii* has also been found in ticks from Germany, Russia, Italy, France, Croatia, Portugal, and Spain (*8*). In Greece, *R. aeschlimannii* has been detected in *H. anatolicum excavatum* ticks collected from sheep (*1*). The tick removed from this patient was *Rh. turanicus*, a species that has been reported in Spain to be infected with *R. aeschlimannii* (*9*).

The first human case of *R. aeschlimannii* infection was identified in a patient who had fever, rash, and an eschar after travel in Morocco (*10*). *R. aeschlimannii* infections in humans have been previously confirmed in South Africa, in Algeria, and in Tunisia (*8*). To our knowledge, human cases of *R. aeschlimannii* infection have not been reported in Europe. Our results emphasize that ticks should be considered as potential vectors for rickettsial infections in humans. We recommend that when one species or serotype of tick-transmitted *Rickettsia* is identified in an area, physicians be informed through established clinical or public health channels of the potential pathogen, its manifestations, and recommended treatments for humans.

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Antibodies against Rift Valley Fever Virus in Cattle, Mozambique

To the Editor: During the past 2 decades, several countries in Africa and the Arabian Peninsula, to which Rift Valley fever virus (RVFV) is endemic, have reported outbreaks of Rift Valley fever in humans and livestock. The first evidence of RVFV in Mozambique was documented as early as the 1960s (*1*). Endemicity was subsequently confirmed in the 1980s by a prevalence study that identified virus-specific antibodies in 2% of pregnant women (*2*) and in the 1990s by serosurveillance in Zambezia Province, which showed that cattle had been infected with RVFV (*3*).

Apart from those observations, the RVFV situation in Mozambique is still poorly understood. We recently found an unexpectedly high level of RVFV activity in cattle in Namaacha District in Maputo Province (*4*), a region where there had been no recorded evidence of the virus since 1969 (*1*). We conducted a cross-sectional study in which serum samples were collected throughout Maputo Province

during 2010–2011 to ascertain whether any RVFV circulation had remained undetected among bovids.

The study was approved by the Mozambican Board of Agriculture. Animals investigated were of mixed breed, had been present in their respective localities since birth, were >6 months of age, and had not been vaccinated against RVFV. Samples were analyzed by using a plaque-reduction neutralization test (*4*), and RVFV seropositivity was defined as 80% reduction of virus infectivity at a serum dilution of 1:40.

A total of 404 serum samples were analyzed, and 149 were positive for RVFV-neutralizing antibodies. This finding represents an overall seroprevalence of 36.9% (95% CI 32.2%–41.6%) in Maputo Province, which is a high level for an area in which no RVFV disease activity has been reported during the past 4 decades.

Although the study was designed to determine the overall prevalence in the province, our data also provided an indication of the distribution of RVFV at a district level. Maputo Province is subdivided into 7 districts, and samples from 6 of these districts were available for analysis: Boane (n = 28), Magude (n = 34), Manhiça (n = 65), Marracuene (n = 82), Matutuine (n = 131), and Moamba (n = 64). The livestock populations in Magude, Manhiça, Matutuine, and Moamba Districts range in size from 20,000 to 70,000 animals, and Boane and Marracuene Districts have smaller populations of 6,000 and 9,000, respectively.

We found the highest seroprevalence to be 61.5% (95% CI 49.7%–73.4%) in Manhiça District and 62.2% (95% CI 51.7%–72.7%) in Marracuene District. Some of the animals affected by RVFV during outbreaks in 1969 were raised on farms near or in these 2 regions (*1*). Our data indicate that the RVFV activity is still high in those districts, possibly because breeding of mosquito vectors is promoted by environmental factors, including irrigation ditches on sugar cane farms, extensive flood plains, wetlands, and ponds (*5*).

South Africa keeps continuous records of RVFV outbreaks, and several outbreaks in cattle were reported in KwaZulu-Natal and Mpumalanga Provinces during 2008–2010 (*6*). Matutuine, the southernmost district in Maputo Province, shares a border with KwaZulu-Natal Province, whereas Magude and Moamba Districts border Mpumalanga Province. Accordingly, the high seropositivity rates of 19.8% (95% CI 13.0%–26.7%), 26.5% (95% CI 11.6%–41.3%), and 29.7.% (95% CI 18.5%–40.9%) in Matutuine, Magude, and Moamba Districts, respectively, are not remarkable. In comparison, a seroprevalence of 14.3% (95% CI 1.3%–27.2%) was noted in Boane District, which has a smaller livestock population.

Because of long-term persistence of IgG against RVFV, the actual time of infection could not be determined from the data obtained in our study. However, information was available regarding the age of the cattle in Moamba District. From this information, we deduced that the most recent RVFV infections in this district occurred at some point during 2009–2010.

Our results strongly suggest that RVFV is widely distributed among bovids in Maputo Province, although the modality of this circulation is unknown. RVFV infection can remain undetected in adult livestock but can cause abortions in pregnant animals and neonatal death in small ruminants (*7*), which has major economic consequences. Transmission to humans is common during epizootics, and the proximity to and density of cattle in an area have been shown to be major factors for RVFV seroconversion in human populations (*8*). Human infections are often manifested as a febrile illness and can easily be mistaken for other diseases. Consequently, unidentified or underdiagnosed RVFV infections among livestock in Maputo Province warrant further research, and implementation of surveillance and livestock vaccination programs in the studied area should be discussed.

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Avian Influenza A(H7N9) Virus Infections, Shanghai, China

To the Editor: On March 31, 2013, the National Health and Family Planning Commission of China notified the World Health Organization of 3 cases of human infections with avian influenza A(H7N9) virus. These cases were caused by a novel virus that was identified by laboratory testing at the China Centers for Disease Control and Prevention (CDC) on March 29 (*1*).

As of April 19, 2013, a total of 91 laboratory-confirmed human cases (17 deaths) of infection with avian influenza A(H7N9) virus were reported in 4 provinces in China (*2*). We report clinical features of 2 infected adults who died, 2 critically ill infected adults who recovered, and 1 infected child who had a mild case during this outbreak in Shanghai, China.

A 3.5-year-old boy had fever (39.5°C) for 3 days and mild rhinorrhea starting on March 31. He was admitted to a district pediatric outpatient clinic on April 1. At admission, the child was given oseltamivir for 5 days, even though signs and symptoms had resolved. Nasopharyngeal swab samples were positive by real-time PCR for avian influenza A(H7N9) virus. All symptoms resolved uneventfully by April 3, and CDC was notified that avian influenza A(H7N9) virus was identified in his respiratory sample. The patient was discharged on day 11 after illness onset.

The 4 adult patients were given diagnoses of severe pneumonia with shortness of breath, dyspnea, and marked hypoxia (Table). Duration from disease onset to severe illness was 5–7 days. At admission, the 4 patients with severe cases had decreased peripheral blood leukocyte counts and increased levels of aspartate aminotransferase; 3 had increased levels of lactate dehydrogenase (Table).

All 4 adult patients had radiologically confirmed pneumonia and bilateral patchy alveolar opacities or diffused lobar consolidation with or without pleural effusion (Figure, Appendix, wwwnc.cdc.gov/EID/article/19/7/13-0523-F1.htm). Findings on chest radiographs for severe cases requiring mechanical ventilation were consistent with those for acute respiratory distress syndrome.

Among the 4 severe cases in adults, a 52-year-old woman (patient 1) and a 49-year-old man (patient 2) died from acute respiratory distress syndrome and multiple organ failure on days 14 and 10, respectively, after disease onset and 1–2 days after progression to respiratory failure. Two other patients showed improvement and were virus negative 6 and 4 days after antiviral treatment. After 23–24 days of treatment in an intensive care unit, the 2 patients with severe cases recovered and were discharged (Table).

The 2 patients who died were given methylprednisolone. Of the 2 patients who recovered, 1 was given a low dose of methylprednisolone for 1 week and the other was not given methylprednisolone. Although it is difficult to assess the role of glucocorticoids in treatment because of limited number of cases, caution is advised because of possible serious adverse events, including death, as reported for human infection with influenza A(H1N1) virus (*4*).

One of the adult patients reported exposure to poultry. The family of the child patient raised chickens and ducks, but these animals had no apparent disease, and cloacal swab specimens were negative for avian influenza A(H7N9) virus. One patient who died (patient 2) had frequent occupational exposure to poultry. Sixteen contacts of the child and 45 contacts of the 4 adult patients were monitored, and routine virologic sampling was performed. One contact (husband of patient 1) of a patient who died (Table) became febrile and was positive for avian influenza A(H7N9) virus on April 12 (day 24 after disease onset for patient 1); as of the date of this report, he was receiving treatment in an intensive care unit. However, it is difficult to tell if this is a case of human-to-human transmission or if both persons were exposed to infectious poultry. All remaining contacts had no symptoms and were negative for virus by PCR.

Several features of this avian influenza A(H7N9) outbreak are distinct from those of previous avian influenza outbreaks. Human infection with this virus showed a case-fatality rate of 18.7% (17/91), but this rate is not as high as that for avian influenza A(H5N1) virus (case-fatality rate 59%) (*5*).

Avian influenza A(H7N9) virus infection seems to cause more severe human illness than do other subgroups of H7 influenza A viruses (subtypes H7N2, H7N3, and H7N7), which are usually associated with poultry outbreaks but cause mild disease in humans. However, infection with avian

Table. Characteristics for 4 patients infected with avian influenza A(H7N9) virus, Shanghai, China*

Chacteristic	Patient 1†	Patient 2	Patient 3	Patient 4
Age, y/sex	52/F	49/M	67/M	65/M
Exposure to poultry	None	Continuous	None	None
Sign or symptom at admission	Fever (40.6°C) for 7 d, cough for 1 d, difficulty breathing starting 7 d after illness onset	Fever (39.8°C) for 3 d, cough for 5 d, difficulty breathing and cyanosis starting 5 d after illness onset	Fever (39.7°C) and cough for 7 d starting 7 d after illness onset	Fever (39.0°C) for 5 d, cough for 2 d starting 5 d after illness onset
Physical examination results	HR 120 bpm, RR 40 breaths/min, BP 140/75 mm Hg, decreased breath sounds, no rales	RR 40 breaths/min, BP 240/160 mm Hg, diffuse moist rales	HR 100 bpm, RR 30 breaths/min, BP 110/78 mm Hg, moist rales mainly in left lung	HR 82 bpm, RR 21 breaths/min, BP 118/74 mm Hg, decreased breath sounds in lower left lung, no rales
Laboratory results				
Leukocyte count, $\times 10^9$/L	3.29	2.9	2.89	3.74
Neutrophils, %	92	69.1	78.6	76.7
Lymphocytes, %	5.5	25.2	15.4	18.2
Platelet count, $\times 10^9$/L	155	71	172	82
AST, U/L	95	258	45	77
LDH, U/L	525	>2,150	209	492
CPK, U/L	351	>1,600	170	1,854
CK-MB, U/L	16	32	7	31
Creatinine, μmol/L	69.7	116.0	84.2	74.3
Medications after hospitalization				
Oseltamivir	Started d 13 after illness onset	None	Started d 11 after illness onset	Started d 10 after illness onset
Antimicrobial drugs	MOX started d 13 after illness onset	MOX started d 10 after illness onset	AZT started d 11 after illness onset, MOX started d 15 after illness onset	CEF started d 11–12 after illness onset, MOX started d 13 after illness onset
Corticosteroids	MEP, 80 mg/d started d 14 after illness onset	MEP, 80 mg/d started d 10 after illness onset	MEP, 80 mg/d started d 11 after illness onset, decreased to 40 mg/d, stopped after 1 wk	None
Immunoglobulin	Started d 13 after illness onset	None	Given d 11–15 after illness onset	None
Other conditions	Diabetes mellitus, surgery for thyroid cancer	Obesity	None	Hypertension
Outcome	Died 14 d after illness onset	Died 10 d after illness onset	Discharged 30 d after illness onset	Discharged 27 d after illness onset

*HR, heart rate; RR, respiratory rate; BP, blood pressure, AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; CK-MB, creatine kinase isoenzyme MB; MOX, moxifloxacin; AZT, azithromycin; CEF, ceftriaxone; MEP, methylprednisolone.
†Data for patient 1 were reported by Yang et al. (*3*) and are included for comparison.

influenza A(H7N7) virus resulted in the death of a veterinarian during an outbreak in the Netherlands (*6*). In the 5 patients reported here, avian influenza A(H7N9) virus caused fatal disease in 2 adult patients 52 and 49 years of age, who had other medical conditions. Older age has been reported to confer higher risk for developing more severe influenza-associated outcomes (*7*).

In conclusion, these cases indicated that avian influenza A(H7N9) virus might not be as virulent as avian influenza A(H5N1) virus in humans. Avian influenza A(H7N9) virus does not appear to cause obvious disease in poultry and causes mild disease in children. More severe disease in adults occurred among those had concurrent diseases or were immunodeficient.

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etymologia

Verona Integron

From the Latin *integrare* (to make whole), integrons are systems for capturing and spreading antibiotic resistance genes among gram-negative bacteria. Integrons were first described by Stokes and Hall in 1989, although they clearly contributed to the first outbreaks of multidrug resistance in the 1950s. The Verona integron was first described in carbapenem-resistant *Pseudomonas aeruginosa* isolated from a patient hospitalized at Verona University Hospital, Verona, Italy. Integrons are ancient structures that have been present in bacteria for millions of years, indicating that bacteria had the means of acquiring and disseminating antibiotic resistance long before humans developed antibiotics.

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The Stealth Virus

Paul Griffiths

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This is a lucid read on a complex virus, cytomegalovirus (CMV), that affects those in all ages of life from the unborn fetus to the elderly pensioner. Griffiths has used one of the many self-publishing, internet-based services to publish and distribute this book via the popular amazon.com and amazon.co.uk websites. All profits from its sale are being donated to the Royal Free Charity, which has supported his research on CMV for many years.

The book is arranged chronologically; the 18 chapters are headed by a range of years, starting with 1910 (Chapter 1) and ending in 2012 (Chapter 18). An interesting approach is used in the Prologue (dated 180 million years ago), where the virus speaks to the reader in the first person. The virus's voice summarizes the most important aspects of its virology and its clinical impact on a largely unsuspecting human population. Several important themes are introduced here: 1) the author's clear views about the relative irrelevance of experiments on mice to the human situation; 2) the missed opportunities that studying CMV earlier could have created by helping the scientific community to understand and more effectively combat other viruses, such as HIV; and 3) how a vaccine approach would be the best way to control this viral pathogen.

The book starts off by discussing other viruses of clinical importance (polio, varicella zoster, rubella, herpes simplex, and HIV), gradually setting the scene for the emergence of CMV. Interspersed between the history of the science and the emerging clinical importance of this virus are patient case histories. At least 1 case is described in each chapter (suitably anonymized with alternative and sometimes memorable names, such as "Stevie Headbanger"), covering the most common clinical CMV scenarios. These include CMV infection and disease in newborns, pregnant women, transplant patients, persons with HIV/AIDS, and other immunocompromised patients. Other cases also demonstrate the virus' impact on otherwise healthy teenagers studying for exams, retirees caring for their grandchildren, and recovering burn and gunshot victims (in this instance, Pope John Paul II). Such case histories provide clear examples of complications arising from both primary and reactivated CMV infection and disease.

Most (if not all) of the relevant major scientific and medical advances related to CMV are noted, including the development of the various anti-CMV drugs, with due credit given to the investigating teams. Most passionately of all, Griffiths carefully charts gradually changing attitudes to the development of a CMV vaccine.

Curiously, the author writes about his own role in the CMV story in the third person—perhaps to maintain a scientist's natural objectivity. Notably, in the latter half of the book, he tends to use more technical jargon, which may cause problems for some readers. Yet, this does not necessarily detract from the book's value; rather, it may provide reassurance to the readers that they are, indeed, in the hands of a master.

The development of a CMV vaccine is now gaining support internationally, thanks in no small part to the efforts of the author. When the vaccine is eventually available, anyone with any doubts about its potential benefits can simply read this little book.

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EMERGING INFECTIOUS DISEASES®

January 2013

Foodborne Infections

Summer Buzz

Polyxeni Potter

"It is late Sunday afternoon in August. A child stands alone in the garden listening to the metallic sounds of insects," reads Charles Burchfield's own description of *The Insect Chorus*. "They are all his world, so, to his mind, all things become saturated with their presence–crickets lurk in the depths of the grass, the shadows of the trees conceal fantastic creatures, and the boy looks with fear at the black interior of the arbor, not knowing what terrible thing might be there."

The Insect Chorus, one of several works on the same theme, was painted during what Burchfield called his "golden year," when he was fresh out of art school and back home in Salem, Ohio, in 1917. The work is a good example of his take on *en plein air* painting. An avid admirer of the outdoors, he immersed himself in it. He liked everything about it, even the annoying parts that most nature lovers merely tolerate for the sake of beauty. His artistic approach was a unique blend of naturalism, emotion, and imagination. "Surrounded by the familiar scenes of my boyhood, there gradually evolved the idea of recreating impressions of that period, the appearance of houses, the feelings of woods and fields, memories of seasonal impressions…."

Nature and its creatures remained of great interest to Burchfield all his life. He felt their sounds and captured them with extraordinary draftsmanship in his trademark watercolors. On the back of *The Song of the Katydids on an August Morning* (1917), he wrote, "A stagnant August morning during the drought season, as the pitiless sun mounts into the mid-morning sky, and the insect chorus commences, the katydids and locusts predominating. Their monotonous, mechanical, brassy rhythms soon pervade the whole air, combining with the heat waves of the sun, and saturating trees and houses, and sky." Of *The August North (A memory of childhood)*, he wrote, "As the darkness settles down, the pulsating chorus of night insects commences swelling louder and louder until it resembles the heartbeat of the interior of a black closet."

"Well, at one time I did think I was going to be a writer–when I was in high school and even the early years in art school I thought I was going to be a nature writer in somewhat the sense, you know, that Teale writes and Harold Borland, and so forth. And if I did anything with art work, I would make my own illustrations. But I soon realized that my outlook was visual rather than otherwise. But I mean it still… I did these short things." He wrote impressions in his copious journals and descriptions on the back of his watercolors: "Crabbed old age sits in front of the black doorway without hope for the future, brooding. Spiders lurk in dark corners. The dying plants reflect her mood. The romantic outer moon rises just the same." He even illustrated his own words, as in his notebook "Conventions for Abstract Thoughts," his personal iconography, "A graphic shorthand of youth," he called it, which came to be viewed by some as a visual vocabulary of American modernism.

Aside from colors and words, other forces also marked Burchfield's output. "I get more out of music probably than

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I do out of other artists' paintings, although I have many great admirations there," he acknowledged. "It seems pertinent to me to insert here some thoughts on how interwoven music is with my painting. To many works, and even for whole periods of time, a definite piece of music or composition seemed to belong, though there might be no connection whatsoever between the music and what I was doing."

Burchfield's sensitivity to all that surrounded him nurtured his imagination and culminated in hundreds of works filled with a vibrant vision of the world, a vision in which humans and their creations were secondary and paled against the overpowering presence of nature. His style engaged whatever tools would bring forward this vision. "There are ideas that come to me that can be interpreted only in terms of patterns, and I derive much pleasure in working them out." He defined his occupation in clear terms, "I like to think of myself—as an artist—as being in a nondescript swamp, up to my knees in mire, painting the vital beauty I see there, in my own way, not caring a damn about tradition, or anyone's opinion."

In *The Insect Chorus*, Burchfield paints summer in the outdoors, seen through the eyes of a child. The psychedelic spectacle transcends visual barriers to express the sweltering heat and humidity, along with the sounds of summer, its mystery and music. The alarming closeness of the clapboard household to the vegetation, which seems possessed by unknown forces, adds to the drama. Drooping ferns frame the canopy and its mysterious depths underneath the cool-color arabesques. Elaborate patterns—fluid lines, broad curves, cones—along with symbols to indicate sound and movement, transform a commonplace scene to a steaming hallucinogenic vision filled with emotion. V shapes in the grass mimic the jagged flight of crickets, and zigzag paths simulate their metallic sound, which Burchfield named "high shrill pin-point cricket chorus."

By the early 1900s, mosquitoes were shown to transmit yellow fever and malaria from person to person, and human malaria was endemic in the United States. When Burchfield's imaginary boy "looks with fear at the black interior of the arbor, not knowing what terrible thing might be there," he is probably not worried about crickets alone but about all manner of vermin that you cannot see yet know are there. In the heat of a "Sunday afternoon in August," mosquitoes awaiting a blood meal would be lurking in the vegetation. Along with a silent chorus of ticks, fleas, and rodents, they are part and parcel of the season and the locale.

Apart from nuisance and noise, the namesake insects of this painting rightly provoke fear. Many human pathogens infecting a large proportion of the world's population and contributing to emerging disease are zoonotic and vector-borne. And many of these viruses, bacteria, protozoa, and rickettsia can be transmitted by blood-feeding arthropods. Depending on the geographic location, Burchfield's vibrating canopy could play host to yellow fever, Rift Valley fever, Lyme disease, tularemia, malaria, typhus, Rocky Mountain spotted fever, to name only a few vector-borne infections that can begin with a summer buzz.

Vectors, such as mosquitoes, bridge barriers that would prevent transmission by direct contact between humans and especially between animals and humans. These barriers are not only spatial but also behavioral and ecologic. This ability of vectors includes moving a pathogen from one region to another. Because of the complex epidemiology and adaptiveness of pathogens and arthropods, vector-borne diseases are difficult to control, much less to eradicate. Vaccines are only available for a few diseases, and even when they are available, as for yellow fever, prevention can still be difficult to achieve.

Even previously successful strategies, such as pesticide impregnated bed-nets, can be compromised by human behavior and vector biology. With malaria, problems must be solved largely on the basis of local data because rarely do variables in one area behave in the same way as in another area, however closely the two regions may seem to resemble each other in topography and climate.

"How slowly the 'secrets' of my art come to me," Burchfield lamented near the end of his life. He might as well have been expressing the epidemiologists' frustration when they realize that changes in climate, land use, and transport, a concert eons in the making, affect rates of pathogen emergence in ways we still do not rightly understand. The artist's respect for the all-encompassing power of nature applies across the board.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

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Emergent Norovirus GII.4 Sydney Strain, United States, 2012–2013

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ESBL- and AmpC-Producing Enterobacteria in Healthy Broiler Chickens, Germany

ESBL-Producing Enterobacteria among Travelers, the Netherlands

Aichi Virus in Sewage and Surface Water, the Netherlands

Accuracy of Diagnostic Methods and Surveillance Sensitivity, South Korea, 1999–2011

Effect of 2003 California Regulation on Raw Oyster-associated *Vibrio vulnificus* Illnesses and Deaths

Macrolide Resistance of *Mycoplasma pneumoniae*, South Korea, 2000-2011

Recombinant Coxsackievirus A2 and Deaths of Children, Hong Kong, 2012

Campylobacteriosis Outbreak among Humans Associated with Duck Liver

Novel G10P[14] Rotavirus Strain, Northern Territory, Australia

Travel-associated Diseases, Indian Ocean Islands, 1997–2010

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Whole Genome Sequencing of Unusual Serotype of Shiga toxin–producing *Escherichia coli*

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Genotype GI.6 Norovirus, United States, 2010–2012

Foodborne Illness Complaint Hotline and Norovirus Surveillance, Minnesota, 2011–2013

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

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Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 70% passing score) and earn continuing medical education (CME) credit, please go to **www.medscape.org/journal/eid**. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the New Users: Free Registration link on the left hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/category/2922.html. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title:

Transmission of *Streptococcus equi* Subspecies *zooepidemicus* Infection from Horses to Human

CME Questions

1. You are seeing a 50-year-old male who works as a horse trainer. The patient has a 2-week history of malaise, back and neck pain, and fever. He had thought he had the flu, but his wife noticed that he was confused and lethargic this morning and brought him to the doctor's office.

You consider whether this patient might have an infection with *Streptococcus equi zooepidemicus. S. zooepidemicus* was associated with all of the following sites of infection in the current case series EXCEPT:

A. Throat
B. Joint
C. Soft tissue
D. Central nervous system/meningitis

2. What should you consider regarding the clinical course of patients with *S. zooepidemicus* infection in the current study?

A. None of the patients had recent exposure to horses
B. All patients died of the infection
C. There was broad antimicrobial resistance of *S. zooepidemicus* isolates
D. All patients had objective findings of systemic inflammation

3. The patient is admitted to hospital, and his blood culture grows *S. zooepidemicus*. What should you consider regarding the transmission of this infection?

A. *S. zooepidemicus* is only found in horses and humans
B. Half of the horses found to harbor *S. zooepidemicus* in the current study were sick
C. Two patients in the current study were infected with the same strain of *S. zooepidemicus* despite living over 100 km from each other
D. All 3 patients in the current study were infected with the same strain of *S. zooepidemicus*

4. As you treat this patient, what should you consider regarding molecular findings from the current study?

A. Gene sequencing failed to connect human with equine isolates
B. Pulsed-field gel electrophoresis profiles were similar for the same strain of *S. zooepidemicus*
C. All multilocus sequence typing (MLST) sequence types correlated to known sequences of *S. zooepidemicus*
D. One strain was similar on MLST to a strain associated with a previous disease outbreak

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

www.cdc.gov/eid

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- ★ New infections resulting from changes or evolution of existing organisms.
- ★ Known infections spreading to new geographic areas or populations.
- ★ Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- ★ Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - ★ Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - ★ Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - ★ Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.